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EDITORIAL

Telomere shortening as genetic risk factor of liver cirrhosis

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Abstract

Cirrhosis is the main complication of chronic liver disease, leads to progressive liver function impairment and is the main risk factor for the development of liver cancer. Liver failure at endstage cirrhosis is associated with increased mortality with liver transplantation as the only possible treatment at this stage. The pathogenesis of liver cirrhosis is not completely elucidated. Although the common factors leading to liver injury, such as viral hepatitis, alcohol consume or fatty liver disease can be identified in the majority of patients a small percentage of patients have no apparent risk factors. Moreover given the same risk factors, some patients progress to cirrhosis whereas others have a benign course, the reason remains unclear. In order to develop new diagnostic and therapeutic tools, it is s essential to understand the pathogenesis of cirrhosis. The identification of genetic risk factors associated with cirrhosis is one of the possible approach to achieve these goal. In the past years several studies have supported the role of telomere shortening and cirrhosis. In the recent year several studies on the relation between several single nucleotide polymorphism (SNPs) and cirrhosis have been published; it has been proposed also a cirrhosis risk score based on seven SNPs. Also epidemiological studies on identical twins and in different ethnic groups have been supporting the importance of the role of genetic risk factors. Finally in the very recent years it has been suggested that telomere shortening may represent a genetic risk factor for the development of cirrhosis.

Key words: Liver cirrhosis; Genes; Single nucleotide polymorphism; Gene mutation; Telomere; Telomerase

Core tip: Common risk factors leading to chronic liver injury can be identified in most patients with cirrhosis, but approximately 5% of patients have no apparent risk factors. Understanding the pathogenesis of cirrhosis formation is essential to develop new diagnostic and therapeutic tools and for its prevention. The identification of genetic risk factors associated with cirrhosis is one of the possible way to approach this issue. Evidence supporting the role of genetic risk factors has been accumulating during the past years and recently it has been also suggested that telomere shortening may represent a genetic risk factor.

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CIRRHOSIS

Cirrhosis represents the main complication of chronic liver disease[1-3], leads to progressive liver failure, eventually to hepatocarcinoma[4] and is associated with increased mortality[5] with liver transplantation as the only possible therapeutic option at this stage. The pathogenesis of liver cirrhosis remains unclear and although the common risk factors, such as viral hepatitis, alcohol and non-alcoholic fatty liver disease (NAFLD) can be identified in the majority of patients, there is a small percentage of patients with no identifiable risk factors[6]. Moreover it is known that patients with the same risk factors present different clinical manifestations, some develop cirrhosis and hepatocarcinoma (HCC) others have a benign course, the reason of the different evolution is not completely elucidated[7]. Understanding the complex pathogenesis of cirrhosis is important for its prevention and to identify new diagnostic tools as well as new therapeutic options; one of the possible approaches to this issue might be to understand the genetic bases of cirrhosis[8,9].

GENETIC CAUSES OF CIRRHOSIS

In the past years several studies have supported the role of telomere shortening and cirrhosis.

In a recent study it was proposed a cirrhosis risk score (CRS) based on a panel of seven single-nucleotide polymorphisms (SNPs) in six genes: *AP3S2*, *AQP2*, *AZIN1*, *STXBP5L*, *TLR4*, *TRPM5* and in the intergenic region between *DEGS1* and *NVL* for identifying the risk of developing cirrhosis in Caucasian patients with chronic hepatitis C infection[10]. The CRS performance resulted even a better predictor of cirrhosis than clinical factors. It is also been utilized and validated in another study on patients with hepatitis C virus (HCV) hepatitis[11], it was observed that host genetics defined by CRS, predict fibrosis progression and may help for prognostic evaluation and treatment decision.

Furthermore, in a study conducted on a large cohort of patients with histologically proven NAFLD it was demonstrated that the *PNPLA3* (patatin-like phospholipase domain-containing protein 3) SNP rs738409 was more frequent in patients with fibrosis[12]. It was also observed by Stickel *et al*[13], that the PNPLA3 SNP rs738409 was more frequent also in Caucasian patients with alcoholic cirrhosis.

Some SNPs have been associated to an increased susceptibility to liver cirrhosis also in HBV infected patients. It has been described a polymorphism of the Glucose-Regulated Protein 78 (GRP78): rs430397 G>A. The Authors observed that HBV infected patients carrying the allele 430397A were more prone to develop liver cirrhosis compared to wild type patients[14].

Taken together these data suggest a genetic predisposition to the development of cirrhosis. The real contribution of these SNPs on the genetic predisposition is not yet elucidated.

Finally data from epidemiologic studies reported difference in prevalence of cryptogenic cirrhosis in different ethnic groups.

A study by Browning *et al*[15] revealed that given the same prevalence of diabetes in Hispanics and African Americans they had a different cryptogenic cirrhosis' prevalence, respectively, 3-fold higher and 4-fold lower compared to European Americans; these finding not only indicate that this form of cirrhosis is unexpectedly rare among Afro Americans, but indicate also that there is a different genetic predisposition among different ethnic groups.

Also studies on twins show the same results. Data on monozygotic and dizygotic twins reported a genetic predisposition in developing alcoholism related disease. In fact it has been shown, for alcoholic psychosis and cirrhosis, a significantly greater concordance in monozygotic twins-pairs compared to dizygotic twins-pairs (16.9% *vs* 5.3%)[16].

TELOMERE SHORTENING AND CIRRHOSIS

In addition to the results from SNP and epidemi-ological studies, recently it has been suggested that telomere shortening may represent a genetic risk factor for the development of cirrhosis.

Telomeres consist of repetitive DNA sequences (TTAGGG) located at the ends of linear chromosomes. They function as a cap to stabilize and protect chromosomes from erosion and from being mistaken for double-strand DNA breaks. During each cell division, telomeres shorten due to the "end-replication problem" that is the DNA polymerase's inability to fully replicate the 3' end of chromosomes. Telomere attrition is avoided by telomerase that elongates telomere DNA after each cell division[17]. In conditions of chronic liver injury there is a high cell turnover as a regeneration and repair process, telomeres become too short and the cell does not divide anymore, the mechanisms by which cell growth arrest is profibrogenic is not yet defined (Figure 1).

Several studies have investigated the relationship between cirrhosis and telomere shortening. Studies on humans showed that shortened telomere length in hepatocytes is correlated with degree of fibrosis and it may be considered as a marker of cirrhosis formation. In 1995 Kitada *et al*[18] demonstrated the relationship between telomere shortening and liver cirrhosis. They observed that telomere length was increasingly shorter in tissue from cirrhotic and chronic hepatitis livers and obviously shorter than normal liver tissue. Following studies confirmed that telomere length was related to the degree of fibrosis, suggesting that it may contribute to the development of cirrhosis[18-20].

Moreover, studies on telomerase-deficient mice provided experimental evidence that shortened telomeres, in response to chronic liver injury, are associated with impaired liver regeneration and accelerated cirrhosis

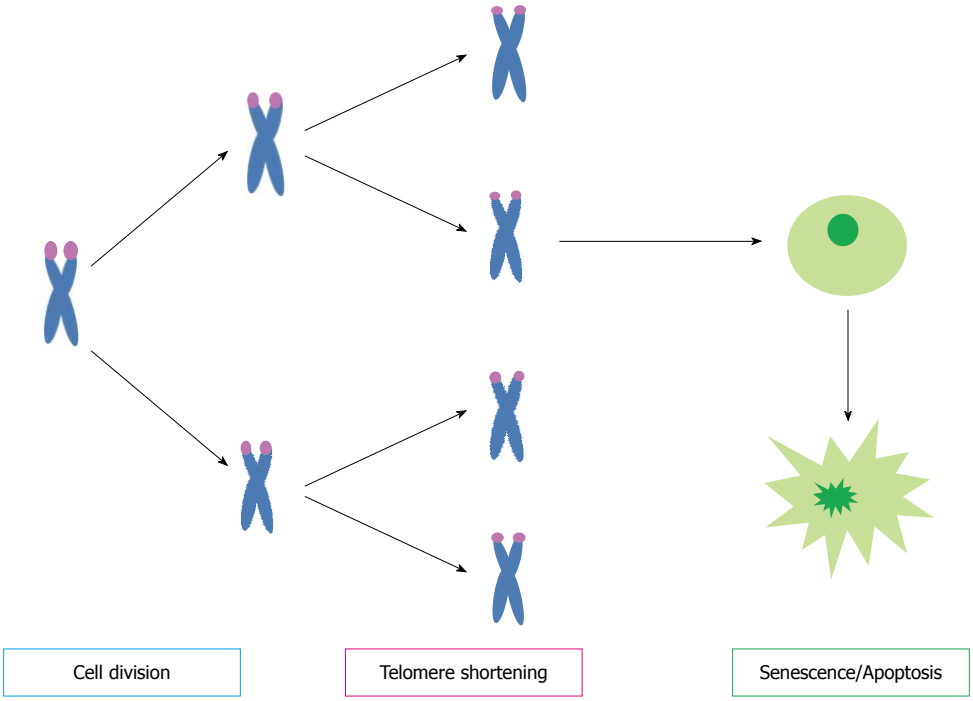

Figure 1 Telomere shorten at each cell division, when they became critically short cellular signaling cascades are activated resulting in cell senescence or apoptosis.

development. Restoration of telomerase activity into the liver of these mice resulted in reduction of cirrhosis and improved liver function[21]. These findings suggest that reduced telomerase activity may contribute to cirrhosis development. Mutations in the telomerase complex genes have been implicated in rare human diseases characterized by accelerated telomere shortening and organ failure such as dyskeratosis congenita. Interestingly, patients suffering from these diseases showed an increased frequency of liver pathologies including fibrosis and cirrhosis[22]. Recently, two studies have also investigated the frequency of telomerase mutations in patients with sporadic cirrhosis compared to healthy controls[21,22]. Both studies screened patients for variation in the *TERT* and *TERC* genes. In the first study by Calado *et al*[23]. The Authors found missense mutations in the *TERT* and *TERC* genes. The frequency of *TERT* gene mutations in cirrhotic patients was significantly greater than controls. Moreover cirrhosis was also associated with shorter telomeres in peripheral blood leukocytes. Finally, most *TERT* variants showed reduced telomerase activity *in vitro*. In the second study Hartmann *et al*[24]. Screened patients with sporadic cirrhosis and noncirrhotic controls for telomerase mutations. Of note, control group was composed of 473 healthy individuals and 127 patients with chronic HCV infection who did not develop cirrhosis during a long follow-up. The data analysis revealed a significantly increased frequency of telomerase mutations in the cirrhosis group compared to the control group. Again, *TERT* gene mutations in cirrhotic patients were associated with reduced telomerase activity *in vitro* and shorter telomeres in peripheral blood leukocytes compared to non-cirrhotic patients. Our group recently reported on the coexistence of cryptogenic cirrhosis (CC) and IPF, not in the setting of dyskeratosis congenita, and telomere dysfunction[25]. In our study was described a case of a 48-year-old woman, diagnosed of cryptogenic liver cirrhosis, idiopathic pulmonary fibrosis and diabetes. Both CC and IPF had a rapid progression and after eighteen months the patient died. Sequencing and mutation analysis of *TERT* and *TERC* genes demonstrated the presence of a heterozygous *TERT* mutation (L153M). The *TERT* L153M variant results in a change of methionine for leucine at amino acid 153, in the protein region that seems to be involved in the template RNA and telomeric DNA binding. Furthermore, leukocyte telomere length was significantly shorter. Our case report gives further evidence of telomere involvement in liver disease progression and suggests short telomere as genetic risk factor for cirrhosis. Moreover very recently our group reported on a similar case of a 58 years old man who presented a short telomere syndrome: pulmonary fibrosis, mild bone marrow fibrosis and a liver cirrhosis. The patient was an active smoker and obese. He rapidly developed ascites and progression of the pulmonary fibrosis, the patient became oxygen-dependent in few months. He presented a very short leukocyte telomere length and reduced telomerase activity compared to a group of controls, but did not show any *hTERT* and *hTERC* genes mutations[26]. Since the prevalence of telomerase mutations seems to be rather low in the general population, they may not be the major contributing factor to cirrhosis. Probably looking for telomerase genes mutations only, there is

the risk to underestimate the real contribution of the telomere system dysfunction to the development of cirrhosis. In fact other components of the telomere complex such as dyskerin and the telomere binding proteins have been shown to be important for telomerase activity[27]. Mutations in these components can lead to an impairment of telomere function; recently a mutation of the binding protein TIN2 has been involved in the evolution of aplastic anemia[28]. Finally also the mutations in the noncoding sequence of *TERC* and *TERC* could be responsible for impairment in the expression of *TERC* and *TERT*. Probably the sequence analysis of all components of the "telomere system or telosome" will reveal the real contribution of telomere complex genes mutations to the development of liver cirrhosis. Together, the current data would suggest that telomere attrition may play a role in the sequence of events leading to cirrhosis. According to this view chronic liver injury induces hepatocyte regeneration and therefore an elevated cell turnover; hence increased telomere shortening and cell senescence. Eventually, if the injury persists, other cells, such as stellate cells become activated leading to fibrogenesis[29]. Therefore telomerase mutations might be considered as genetic risk factors for cirrhosis and telomere shortening as an important step in its pathogenesis. A clinical implication of these findings would be the future use of assay of telomerase gene mutations in the selection and treatment of patients with liver disease[7]. What is the relationship between cirrhosis, HCC and telomere complex dysfunction? Since carcinogenesis reactivate telomerase activity, it is not clear yet if patients with telomerase mutations have a greater predisposition to develop HCC after cirrhosis[7]. More data and larger studies are needed to understand the real impact of telomere dysfunction on HCC development. It is not known; neither is clear why some patients with cirrhosis will develop HCC relatively early in the natural history of cirrhosis whereas others with more advanced stages of liver dysfunction do not develop HCC; if this could be related to telomere dysfunction needs to be investigated. Future studies with large number of patients are needed to provide answers to these questions.

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REVIEW

Role of the diet as a link between oxidative stress and liver diseases

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Abstract

Oxidative stress is caused by an imbalance between the production of reactive oxygen (free radicals) and the body's ability (antioxidant capacity) to readily detoxify the reactive intermediates or easily repair the resulting damage. An adequate diet, characterized by daily intake of foods associated with improvements in the total antioxidant capacity of individuals and reduced incidence of diseases related to oxidation, can modulate the degree of oxidative stress. In fact, diet-derived micronutrients may be direct antioxidants, or are components of antioxidant enzymes, leading to improvement of some indicators of hepatic function. However, although their increased dietary intake might be beneficial, literature data are still controversial. This review summarizes what is known about the effects of diet nutrients on oxidative stress, inflammation and liver function. Moreover, we have analyzed: (1) the main nutritional components involved in the production and/or removal of free radicals; and (2) the role of free radicals in the pathogenesis of several hepatic diseases and related comorbidities.

Key words: Nutrition; Micronutrients; Macronutrients; Liver disease; Oxidative stress

Core tip: Nutritional intake is a fundamental determinant of health. Recently, it has been observed that dietary supplementation has hepatoprotective and anti-oxidant effects. The aim of this review was to summarize the molecular changes promoted by diets and to underline the relationship between diet, oxidative stress and liver disease.

Arrigo T, Leonardi S, Cuppari C, Manti S, Lanzafame A, D'Angelo G, Gitto E, Marseglia L, Salpietro C. Role of the diet as a link between oxidative stress and liver diseases. *World J Gastroenterol* 2015; 21(2): 384-395 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/384.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.384

INTRODUCTION

Dietary elements have long been known to play a critical role in the physiological or pathological response to tissue inflammation and oxidative stress (OS). Accordingly, deficiency or excessive production of most dietary nutrients can impair the balance between the anti- and pro-oxidant agents, also causing liver diseases. Although few studies have evaluated the histological signs in hepatic diseases after adequate and/or inadequate dietary intake[1,2], the involvement of nutrition and OS in the pathogenesis of liver dysfunction has been assessed[1,3,4]. In fact, specific micro- or macro- nutrients may play a critical role in liver cell integrity, influencing fibrosis, inflammation, and degree of liver steatosis caused by OS. Therefore, it is useful to understand the underlying mechanism for the production and removal of free radicals (FR) and/or other reactive species. FRs, including superoxide anions, hydroxyl radicals, and hypochlorous acid, show "two faces" in biology. They may be released in the liver as a consequence of physiological (*e.g.*, signal transduction, gene transcription, phagocytosis, hepatic detoxification, and metabolic pathways) and pathological conditions (*e.g.*, inflammation and necrosis)[5-7]. The impairment of antioxidant defense mechanisms (*e.g.*, superoxide dismutase, catalase and glutathione peroxidase, and glucose 6-phosphate dehydrogenase) could permit enhanced free radical-induced tissue damage. Therefore, reactive oxygen species (ROS)-mediated injury to membranes, proteins, DNA and RNA, generation of pro-inflammatory cytokines, activation of spindle cells, and finally fibrogenesis can occur[8,9]. The main targets of OS are the endoplasmic reticulum (ER) and mitochondria.

The ER regulates the synthesis and release of membrane proteins. The maintenance of its function requires high concentrations of intra-ER Ca^{2+}. Several injuries induce a decrease in physiologically high intra-ER Ca^{2+} levels that result in impaired ER function, also known as "ER stress", promoting apoptosis, hepatic stellate and Kupffer cells recruitment, and synthesis of inflammatory cytokine-inducible factors[10].

Although mitochondria can control the oxidative balance, under continuous stressful stimuli they collapse and become producers of oxidative damage[11]. In fact, mitochondrial fatty acids are normally transferred to the β-oxidation pathway. Fatty acid overload can lead to an imbalance between increased delivery of electrons to the respiratory chain and their decreased outflow from this chain, causing accumulation of ROS and peroxidation products. Thus, mitochondria represent the main source of ROS. Furthermore, ROS attack mitochondrial DNA and promote accumulation of DNA mutations, which, in turn, lead to further ROS synthesis[11]. Additionally, by inducing collagen gene expression, lipid peroxidation products can stimulate fibrogenesis and apoptosis. The released apoptotic bodies can activate hepatic stellate cells and convert them, through transforming growth factor β1, platelet-derived growth factor and endothelial growth factor, into myofibroblasts. Moreover, apoptotic bodies can also stimulate Kupffer cells to generate ROS, which further enhance apoptosis, and the release of cytokines and chemokines that contribute to the activation of hepatic stellate cells into myofibroblasts[12]. Myofibroblasts, through nicotinamide adenine dinucleotide phosphate, can also induce the ROS synthesis, and the release of adhesion molecules for T lymphocytes and natural killer T-cells. Finally, myofibroblasts generate an extracellular matrix resistant to degradation mediated by metalloproteinases[13].

The consequence of these numerous interactive cellular and molecular pathways is to perpetuate and to enhance hepatic tissue injury. Specific dietary nutrients can interfere in the aforementioned pathways, potentially modulating the conversion of highly reactive FR to relatively inert radicals.

NUTRITION AND HEPATIC OXIDATIVE STRESS

Literature data support the usefulness of anti-oxidant activities of micro- and macro- nutrients to prevent many human diseases (*e.g.*, neoplasia, inflammation, autoimmune diseases). On the other hand, growing evidence suggests that hypo- and hyper-nutrition are associated with a higher likelihood of cellular oxidation[14], mediated by specific nutrient pathways.

Here, we discuss the role of micro- and macro- nutrients intake on the development hepatic disease.

Proteins

Proteins, including amino acids (*e.g.*, arginine, citrulline, glycine, histidine, and taurine), small peptides (carnosine), and nitrogenous metabolites (*e.g.*, creatine and uric acid) directly scavenge ROS and also inhibit inducible-nitric oxide synthase (iNOS) expression in various cell types, including hepatocytes[15]. However, when the capacity of this antioxidant system decreases, the level of inactivated ROS rises. ROS-mediated modification might alter both protein structure and function. OS may induce reversible and irreversible changes in proteins. Reversible alteration, generally involving cysteine, can modulate the function of a protein. Irreversible modification, usually lysine, results in a permanent loss of function and may contribute to the degradation and the accumulation of proteins into cytoplasmic inclusions[16]. Moreover, oxidized proteins are highly susceptible to proteolytic attack by proteasomes. Thus, a dietary deficiency of protein is associated with decreased synthesis of antioxidant enzymes and an increased superoxide anion release. Likewise, protein malnutrition can also cause steatosis, modulating the expression of lipolysis and lipid utilization genes in liver[17]. Proteins are involved in several mechanisms of hepatic fat storage. In fact, an adequate protein diet was associated with: (1) major metabolic rates and mitochondrial oxygen consumption; (2) increased β-oxidation of fatty acids; and (3) elevated bile acids levels which, in turn, inhibit lipogenesis[18].

Finally, proteins in the hepatic tissue, produce a large amount of energy.

In animal models, it has been reported that insufficient protein intake causes a deficiency of micro-nutrients (zinc) and serum albumin levels[14,19]. In fact, plasma free iron values are elevated in malnourished patients. Some studies suggested that the increased tissue free iron concentrations likely result from low concentrations of hepatic iron-binding proteins (ferritin, transferrin, and lactoferrin)[20].

An increment in dietary protein content has been noted to prevent the likelihood of liver fat accumulation in excessive fat intake in humans[21].

However, recently, an increased protein intake has been shown to stimulate the generation of ROS, and lipid peroxidation in human polymorphonuclear leukocytes and mononuclear cells[22].

For these reasons, a high-protein and low-carbohydrate diet is not typically used in children because of their low energy intake, which might compromise the child's growth[23]. However, this type of diet is preferred, for a short time, to induce a rapid weight loss in obese children affected by non-alcoholic fatty liver disease (NAFLD)[23].

In conclusion, although the exact mechanism remains to be elucidated, an adequate quality and quantity of protein intake might be helpful in fatty liver disease.

Carbohydrates

Epidemiologic studies have implicated foods containing high concentrations of carbohydrates in the etiology of liver diseases. In fact, high carbohydrate intake, by activation of specific transcription factors, promotes hepatic steatosis and insulin resistance. The most common simple carbohydrates, glucose and fructose, can result in relatively high glycemic index values. High glycemic index foods stimulate excessive and prolonged insulin secretion, which increases deposition of fats and leads to major serum non-esterified free fatty acid values into circulation and in the hepatocytes mass. According to these findings, subjects affected by hepatic disease should avoid high carbohydrate intake, especially fructose[24], which, by inducing depletion of adenosine triphosphate (ATP), causes arrest in protein production, favors inflammatory proteins release, alters endothelial function, and stimulate OS[25]. Several adult studies have found links between higher consumption of total carbohydrates and nonalcoholic steatohepatitis (NASH) and the metabolic syndrome. Although fructose's role in human NAFLD is unknown, it is hypothesized that in addition to OS, lipid peroxidation, cytokine activation, nitric oxide (NO) and ROS, endogenous toxins of fructose metabolites can further lead to hepatic fat accumulation[26]. Fructose promotes intestinal permeability leading to portal overload of endotoxins, pro-inflammatory factors [tumor necrosis factor-alpha: TNF-α)], and fatty acids in the liver[27].

Lower serum high-density lipoprotein levels and higher triglyceride values were previously associated with major fructose intake in children affected by NAFLD[27]. Moreover, mineral deficiency, metabolic imbalance, and higher release of ROS can further favor fructose-induced NAFLD[27]. Recently, a study in normal-weight and overweight children showed that total fructose intake was the only dietary factor that significantly predicted low-density lipoprotein particle size[28].

However, it has been also reported that changes in the quality of carbohydrates intake did not influence hepatic functionality. In fact, isocaloric exchange of fructose for other carbohydrates seems not induce NAFLD changes in healthy subjects[29].

Lipids

Fat is an important component of the normal human diet. It is a source of energy and provides essential fatty acids and fat-soluble vitamins. However, saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs) can have adverse effects on human health. Although SFAs promote OS-resistant states in the liver and protect it against OS[30], studies have demonstrated that SFAs are more toxic than unsaturated fats. High SFA intake leads to a proinflammatory status resulting from an imbalance in lipid signaling pathways and increased production of inflammatory cytokines (*e.g.*, TNF-α, IL-6)[31]. Moreover, SFAs promote endoplasmic reticulum stress as well as hepatocyte injury. In fact, accumulation of SFAs in the liver leads to impaired mitochondrial metabolism and increased markers (ROS) related to endoplasmic reticulum stress. This latter, in turn, contributes to hepatic apoptosis progression[32].

The accumulation of SFAs in the hepatocytes can promote apoptosis by intrinsic and extrinsic pathways. The intrinsic pathway of cell death includes: (1) ROS-induced stress that affects the endoplasmic reticulum, mitochondrial membranes, and lysosomes; and (2) lipid peroxidation that increases the serum ROS levels[33]. The extrinsic pathway is mediated by death ligands, such as Fas (a key death receptor belonging to the TNF-receptor family) and TRAIL (TNF-related apoptosis-inducing ligand), which subsequently stimulate TNF-α production. TNF-α consequently induces the upregulation of pro-apoptotic molecules and, finally, cell death[34].

If overeating SFA promotes hepatic damage and visceral fat storage, an excess energy from PUFA may contribute to perpetuate liver injury. PUFAs are essential fatty acids, which are crucial for normal growth and health, and are not synthesized in the body of mammals. PUFAs exhibit anti-inflammatory action by suppressing pro-inflammatory cytokine production, macrophages and hepatocytes[35]. ω-6 PUFAs (*e.g.*, linoleic acid, alpha-linoleic acid, and arachidonic acid) contribute to the regulation of fatty acid synthesis and oxidation in the liver. However, high intake of ω-6 PUFAs may increase lipid peroxidation, iNOS expression, FR production and oxidative DNA damage in many cell types such

as macrophages, muscle and liver cells. Higher fatty acids levels in portal or systemic circulation promote visceral and hepatic fat deposits, mediated by decreased synthesis of lipoproteins and export of lipids from the liver. Subsequently, liver fatty infiltration may also cause decreased fatty acid oxidation and hepatic steatosis[36]. ω-3 PUFAs (*e.g.*, α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid) inhibit lipogenesis and stimulate fatty acid oxidation in the liver[23]. In rodents, ω-3 PUFAs may influence body composition and obesity. Major intake of ω-3 PUFAs may decrease fat accumulation, mainly visceral fat, and reduce body weight when already obese[37]. It has been suggested that ω-3 PUFAs may activate a metabolic change in adipocytes, including increased β-oxidation, suppressed lipogenesis, decrease fat accumulation and higher apoptosis. Additionally, ω-3 PUFAs seem to reduce prostaglandin synthesis, even more than ω-6 PUFAs[38].

Liver-specific responses, such as regulation of blood glucose homeostasis, sinusoidal blood flow within the liver, properties of the trans-endothelial barrier within the liver, synthesis and release of important other mediators like cytokines, growth factors or NO, and liver fibrogenesis, are mediated or regulated by prostaglandin E2 (PGE2)[39]. Appropriate PGE2 levels are generated through the activation of constitutive cyclooxygenase-1 (COX-1) in hepatocytes. PGE2 plays a crucial role in liver pathophysiology *via* essentially hepatoprotective functions, such as inhibiting the generation of ROS, preventing leukocyte migration, improving hepatic insulin and lipid metabolism and regulating the production of inflammatory cytokines[40]. On the other hand, PGE2 also induces the expression of inflammatory cytokines, which can, in turn, enhance the production of ROS[41].

Previously, Mater *et al*[42] suggested that ω-3 PUFAs combines with the specific site on the COX-1 enzyme that converts ω-6 PUFAs into prostaglandins. Moreover, they can also act as the precursor of prostaglandins; however, their activity is 2-50 times lower than the prostaglandin produced by ω-6 PUFAs. It has also been observed that ω-3 PUFAs influence the synthesis of inflammation resolution mediators by neutrophils[43]. In animals, the anti-inflammatory effect was correlated with overexpression of antioxidant genes (glutathione-S-transferases, uncoupling protein-2 and Mn-SOD)[44]. In humans, however, data are controversial. An insufficient ω-3 PUFAs intake was promotes susceptibility to hepatic inflammation in children with NAFLD[45]. Although it has been reported that ω-3 PUFAs ameliorate hepatic circulation, mediated by suppression of local and systemic proteins, including high-mobility group box 1 (HMGB1)[46-48], an adequate dietary intake of ω-3 PUFA did not reflect systemic pro-inflammatory cytokines and protein levels[49].

It has been reported that diet integration with ω-3 PUFAs might facilitate hepatic metabolic adaptation from in utero nutrition to the postnatal diet, by increasing fatty acid oxidation and modifying glucose and amino acids to anabolic pathways[50]. In addition to normocaloric/normolipidic diet, ω-3 PUFAs treatment correlated with the best metabolic parameters results (lower rise of serum triglycerides, glycemia, and cholesterol levels in serum) and reversed the liver histopathological results[51]. Recently, Chahal *et al*[37] assessed that ω-3 PUFAs supplementation was not significantly effective in treating hypertriglyceridemia in pediatric patients. Although the mechanism remains unclear, it has been proposed that ω-3 PUFAs, in addition to total parenteral nutrition (TPN) and weight reduction therapy[52], reverse or improve abnormal liver tests in children[53]. To date, studies are insufficient regarding the types, amount, and duration of the intake of PUFAs in children[54].

Vitamins

Many vitamins play an important role in preventing radical induced cytotoxicity by inhibition of iNOS activity, its gene transcription and NO production. Vitamins also directly scavenge ROS and upregulate the activities of antioxidant enzymes[15].

Among them, vitamin E (α-tocopherol) is thought to be one of the most important micronutrients that inhibit ROS-induced release of lipid peroxyl radicals, protecting cells from pro-oxidants and OS. Vitamin E, acting as a chain-breaking antioxidant, prevents the propagation of FR in membranes and in plasma lipoproteins. When peroxyl radicals are formed, these quickly react with vitamin E (Vit EOH). The hydroxyl group of tocopherol reacts with the peroxyl radical to form the corresponding lipid hydroperoxide and tocopheryl radical (Vit E-O). This latter, by binding vitamin C, returns vitamin E to its reduced state[55]. The interaction of vitamins E and C has led to the hypothesis of the "antioxidant network", also knows as "vitamin E recycling", by which the antioxidant function of oxidized vitamin E is continuously restored by other antioxidants[56]. During phlogosis, this mechanism also reduces mast cell activation. Mast cells are activated by oxidized lipoproteins, resulting in increased expression of inflammatory cytokines and suggesting the reduction of oxidation of low-density lipoprotein by vitamin E[57]. Therefore, a dietary deficiency of vitamin E reduces the activities of antioxidant enzymes (such as liver GSH peroxidases, glutathione reductase, and catalase) and leads to increased hepatic lipid peroxidation. Fortunately, all these negative effects can be reversed by dietary vitamin E supplementation[58]. However, the efficacy of vitamin E remains controversial in the treatment of liver diseases, including NAFLD. Several experimental studies assessed the potential protective role of vitamin E and showed that it improves the clinical symptoms of NAFLD[59], enhances glucose metabolism[60], and correlates with lower serum transaminase levels[61]. Moreover, Vitamin E, decreases histopathological damage (including degree of steatosis, inflammation and fibrosis) in adults and

children[62-64]. However, other authors did not confirm these data[65-67].

Currently, randomized controlled trials in children have not demonstrated uniformly the beneficial effects of vitamin E on the long-term outcome of NAFLD patients. The TONIC trial did not find a significant correlation between the vitamin E and control groups in improving serum alanine aminotransferase levels, steatosis or hepatic inflammation. However, increased resolution of hepatocyte ballooning was observed in the vitamin E subjects[64].

In humans, vitamin C, a water-soluble electron donor, plays a protective role against FR-induced OS. Vitamin C inhibits peroxidation of membrane phospholipids and acts as a scavenger of FR (superoxide, singlet oxygen, and hydroxyl radicals) and is also required for one-electron reduction of lipid hydroperoxyl radicals *via* the vitamin E redox cycle[67]. Vitamin C prevents hepatic storage of 8-hydroxydeoxyguanosine, a marker of DNA injury, and hinders hepatocellular growth. Moreover, vitamin C arrests bacteria internalization and translocation by increasing the transepithelial membrane resistance[68] and enhancing the ability of neutrophils to kill bacteria. In the presence of bacteria, ascorbate levels in neutrophils increase to protect these cells against damage by ROS that they previously produced[69].

In an experimental model, ascorbate's anti-fibrotic action, attributed to decrease in the oxidative stress, hepatic stellate cells activation, cytotoxicity and mRNA expression of fibrotic genes, has been also been reported[70]. The aforementioned positive effects of vitamins could provide a rationale for dietary vitamin C intake in individuals. However, the supplementation of vitamin C as further liver treatment is still controversial. A Cochrane database meta-analysis, analyzing six trials that used a combination of selenium, vitamin C and vitamin E to evaluate their effects on the NAFLD, found no evidence to support or refute them as useful treatments[71]. Intervention studies with vitamin C have shown no change in markers of oxidation or in clinical benefit. In fact, in children, authors reported no additional effects of using vitamin C or E on weight loss and a possible histological improvement in patients affected by NAFLD[1,72]. Perhaps these controversial data are related to different administration doses. Dose concentration studies of vitamin C in healthy people showed a sigmoidal relationship between oral dose and plasma and tissue vitamin C concentrations. Hence, optimal dosing is critical for intervention studies using vitamin C[73].

Vitamin B_{12} is an essential cofactor that plays important roles in one-carbon metabolism, which is required for the maintenance of intracellular DNA synthesis and methylation. In fact, vitamin B_{12}, serving as a cofactor for methionine synthase, cystathionine synthase, and cystathionase, and as a substrate (5-methyltetrahydrofolate) for methionine synthase, helps to reduce the risk of OS-mediated homocysteine[74]. The liver is the principal storage site of vitamin B_{12}. Consequently, serum Vitamin B_{12} levels reflect liver function. In fact, it has been reported that vitamin B_{12} and hepatic enzyme serum levels are correlated, especially in alcohol-dependent liver disease[74].

Vitamin B_{12}, through epigenomic mechanisms related to imbalanced acetylation/methylation, influences cell proliferation, differentiation and apoptosis. In addition, vitamin B_{12}, by ER stress stimulation, impairs fatty acid oxidation and energy metabolism in the liver[75]. Therefore, increased serum vitamin B_{12} levels have been attributed to the release of the vitamin from the liver during hepatic necrosis and decreased hepatic synthesis of transcobalamins Ⅱ, an essential element for tissue binding of vitamin B_{12}[75].

Minerals

Studies on most micronutrients are complicated by the fact that the nutrients have several roles.

Selenium, a cofactor of numerous enzymes (*e.g.*, glutathione peroxidase, selenoprotein P, and other selenoproteins) has a protective role against peroxidative and/or FRs damage and mitochondrial dysfunction[76]. In humans, deficiency of selenoproteins causes liver necrosis and hepatic cell death by OS. Furthermore, low serum selenium levels have been found in patients with chronic liver disease[77].

Magnesium is involved in ATP-mediated reactions. Deficiency of dietary magnesium reduces glutathione reductase activity, and results in generation of ROS and increased susceptibility to lipid peroxidation, and marked lesions in tissues (*e.g.*, skeletal muscle, brain, and kidney)[78].

Manganese is a component of several enzymes involved in fatty acid and cholesterol biosynthesis, as well as mitochondrial pathways. Manganese is a cofactor for a number of enzymes important for intermediary metabolism, including the hepatic urea cycle enzyme arginase. There are few well-described cases of manganese deficiency in the medical literature[14] and how manganese affects normal metabolism in the liver remains unclear[79].

Copper, zinc and manganese are indispensable metals for the activities of Cu, Zn-SOD and Mn-SOD, respectively[80].

The liver plays an important role in the disposition of copper, which can be used for protein and energy production. Consequently, abnormal copper metabolism can also cause oxidative damage and hepatotoxicity. Mitochondria are the first responders involved in copper homeostasis. In an animal model, Cu^{2+} induced a concentration and time-dependent rise in mitochondrial ROS formation, lipid peroxidation, cytochrome c expulsion, mitochondrial swelling and collapse, and finally cell death signaling. Interacting with respiratory complexes (Ⅰ, Ⅱ, and Ⅳ), Cu^{2+} caused decreases the ATP concentration

and the ATP/ADP ratio mitochondria, favoring liver toxicity[81]. Synthesis of ROS, tissue failure and hepatocyte death are also observed in human disease progression, such as un Wilson disease (WD). Liver damage in WD appears to involve two different pro-oxidants mechanisms: synthesis of ROS, mediated by the Haber-Weiss reaction[82], and apoptosis through the activation of acid sphingomyelinase and consequent production of ceramide[83,84].

The liver plays a central role in zinc homeostasis, removing it from albumin in the blood and distributing it to the body as needed. Zinc acts as an essential cofactor for enzymes that are necessary to counteract hepatic OS[85]. Cirrhotics show increased urinary zinc loss and can become zinc deficient[77].

Liver is an important site for iron, a cofactor for important biological biochemical reactions, including the transport of oxygen and electrons *via* cells, oxidative phosphorylation, energy production, DNA synthesis, cell growth or apoptosis and gene expression[20]. However, iron's bioavailability is limited because its accumulation can enhance OS and related toxic effects. Therefore, iron deficiency and overload are generally regarded as causes of diseases involving OS and lipid peroxidation[86]. Patients with iron deficiency anemia are more sensitive to agents that induce OS[87,88]. On the other hand, daily iron supplementation resulted in increased lipid peroxidation and abnormal iron accumulation; although intermittent supplementation (once every 3 d) alle-viated these effects[89]. It is thought that iron overload, mediated by mobilization of peripheral fat to the liver and development of hyperinsulinemia, promotes insulin resistance. Therefore, the syndrome of "insulin resistance-associated iron overload" was hypothesized in the presence of unexplained hepatic iron overload in patients with insulin resistance[90].

Increased iron storage has been linked with more advanced stages of NAFLD, *via* increased inflammation and oxidative stress[91].

Increased iron accumulation in NAFLD might be the results of decreased serum iron export protein levels, ferroportin, induced by the decrease of the iron regulatory peptide, and hepcidin. Generally, synthesis of hepcidin is upregulated by phlogosis and increased iron stores. Hepcidin in turn leads to the degradation of ferroportin and iron release[92]. In patients with NAFLD, increased expression of hepcidin and lower expression of ferroportin could favor iron accumulation and, consequently, OS and inflammation[93]. Iron overload is also very common in many types of non-biliary cirrhosis, and in end stage liver disease, including hemochromatosis. When liver iron overload is excessive, OS, involving production of iron catalyzed oxygen radicals, represents the main mechanism of liver injury, DNA alterations, and higher risk of cancer in patients affected by hemochromatosis[94].

Although the degree of necro-inflammation did not differ, iron stores were reported to be also elevated in patients with chronic hepatitis C compared to those with chronic hepatitis B[95]. HCV infection is probably associated with increased hepatic iron concentration and deposition (Kupffer cells and portal macrophages), higher serum transferrin saturation, and serum ferritin levels. Additionally, serum iron values were reported to be correlated with progression of liver disease and degree of hepatocyte necrosis[96]. Moreover, iron overload can activate hepatic stellate cells and promote the synthesis of collagen, contributing to hepatic fibrogenesis[97].

Probiotics

Probiotics, also known as "good bacteria" or "helpful bacteria," are live microorganisms (*e.g.*, bacteria), which are beneficial to health and are either the same as or similar to microorganisms found naturally in the human body (Lactobacillus or Bifidobacterium)[98].

Probiotics play a pivotal role in NAFLD and NASH, obesity-related hepatocarcinogenesis, alcohol-related disorders, portal hypertension and obstructive jaundice[6,99,100].

Patients with hepatic diseases showed an impaired gut-liver axis, which contributes to increase blood levels of endotoxemia (lipopolysaccharides, LPS) and chronic low-grade inflammation. Generally, LPS disseminates into the systemic circulation in two different ways: *via* a portal vein or through the lymphatic system. Underlying liver disease caused patients to report an increased gut permeability which promotes bacterial overgrowth and translocation, and increased expression of pro-inflammatory molecules (iNOS, ROS), which in turn promote major gut and sinusoidal permeability, increased pro-inflammatory cytokines and cells (*e.g.*, neutrophil), and mitochondrial damage[7,99-101].

The use of probiotics to manage liver injury is attributed to a variety of their health benefits. Probiotics, which increase cellular permeability and compete with pathogens for binding, improve colonization resistance to gut pathogens by reinforcing the mucosal barrier and restoring normal gut micro-ecology[102,103]. Probiotics can activate and modulate the immune system, and reinforce gut defense by immune exclusion, elimination and regulation[104,105]. The link between gut microbiota, liver inflammation, and immune system involves toll-like receptors (TLRs), which are important mediators between the environment and the immunological response[106] and endogenous substances, such as short-chain fatty acids and HMGB1. TLRs involved in the pathogenesis of NASH are TLR2 (for lipoproteins and glycolipids in bacteria adhering to myeloid dendritic cells, mast cells or monocytes), TLR4 (for palmitic- stearic and lauric-acid, and LPS of B cells myeloid dendritic cells, mast cells, monocytes and intestinal epithelium) and TLR9 (for unmethylated CpG DNA-bacterial particles)[100,107].

Additionally, probiotics reduce hepatic triglyceride contents, ameliorate adipose tissue inflammation[108],

and induce anti-oxidative enzymes that prevent the progression of NASH to hepatocellular carcinoma[58]. In animal models with portal hypertension, by reducing bacterial translocation, probiotics decreased the OS and/or increase vasodilator factors leading to improved endothelial dysfunction in the mesenteric artery[109]. Moreover, an experimental study investigated the role of probiotics in obstructive jaundice. The authors reported that probiotics (*Lactobacillus plantarum*), by activating the protein kinase C pathway, could decrease intestinal epithelial cell apoptosis, reduce OS, and prevent tight junction disruption in biliary obstruction[101].

Although, Cochrane meta-analysis[110] did not approve or refute the use of probiotics as a therapeutic option for patients with NAFLD/NASH, several recent studies showed encouraging preliminary results[111,112]. Compared with controls, patients with liver diseases had initially decreased *Bifidobacterium* and *Lactobacillus* levels and their serum ALT, AST and GGT values were elevated significantly. After treatment, the group who received probiotics had significantly increased *Bifidobacterium* and *Lactobacillus* levels and decreased serum liver enzyme levels compared with patients receiving the placebo[113]. This evidence confirmed both gut-liver axis malfunction and the possible useful role of probiotics in the treatment of liver diseases.

TPN AND HEPATIC OXIDATIVE STRESS

TPN is life saving in patients with clinical problems that preclude enteral diet for a long period. However, long-term TPN-related complications, especially liver dysfunction, have been reported and confirmed by a biological significant increase of cytolysis[114,115]. Several possible mechanisms of TPN-induced liver dysfunction have been hypothesized. Firstly, TPN promotes alteration of some trace elements in hepatocytes. Depletion in the hepatic copper concentration might cause a decrease in the activity of antioxidant and detoxifying enzymes. A parallel depletion of zinc, a cofactor of tissue matrix metalloproteinase that degrades collagen, could induce the accumulation of the extracellular matrix, finally leading to the development of hepatic fibrosis and cirrhosis[116,117].

Secondly, TPN administration favors hepatic lipid accumulation, especially in children[118]. In addition to increased lipid synthesis, accelerated mobilization of fat deposits, impaired fatty acids oxidation and lipid accumulation leads to steatosis, mitochondrial and ER damage, activation of caspases, and consequent Fas ligand/TNF-α-mediated apoptosis. TPN also causes the depletion of carnitine, a compound necessary for the transfer of free fatty acids from the hepatic cytoplasm into the mitochondria. The cytosolic concentration of choline, a nutrient for lipoprotein release, also decreases, promoting lipid storage in hepatic cells[119,120]. Moreover, TPN favors lipid peroxidation by providing PUFAs and perpetuating lipid peroxidation[121].

Thirdly, TPN might lead to impairment of the gastro-intestinal immune system and the development of infections. The factors facilitating this process include: (1) abnormal proliferation and translocation of bacteria[122]; (2) decreased neutrophilic opsonic activity[123]; (3) atrophy of intestinal mucosa and related reduction of serum IgA levels; and (4) reduction of T-helper and IL-2 producing cells[124].

Finally, light exposure to nutrient mixtures affects hepatobiliary responses and histological changes. When TPN was administered intravenously, no damage was noted. Additionally, the severity of TPN-liver damage could be correlated with the duration of TPN administration[125].

In contrast to these data, an observational study assessed that, although the TPN-group showed some signs of increased OS, there were no signs for oxidative damage, compared with control-group. Moreover, the activity of the underlying disease was not correlated to increased OS[126].Other authors confirmed that an increase in OS bio-markers are not necessarily related to the route of pharmaconutrition (TPN); however, it might occur independently[127].

In the light of these data, to reduce the risk of liver disease related to TPN, several management strategies have been proposed recently, including reformulation of standardized parental nutrition. It has also been proposed that an optimal dose and type of parental lipid should be provided to minimize hepatic injury[128].

CONCLUSION

Although it is very difficult to correlate the biochemistry of a dietary intake with the pathophysiology of hepatic disease, diet significantly attenuates the relationship between OS and liver inflammation. In fact, dietary interventions may reduce the impact of hepatic diseases and could be useful in the treatment and prevention of progression to more severe disease. However, although the anti-oxidant properties of the diet are known, investigations into the relationship between the diet and OS are still limited, and most studies were conducted on a single diet element. We believe that the evaluation of combined and parallel roles of nutrients on inflammation and OS might be more helpful. Additionally, the potential correlation between diet nutrients and serological, histopathological, and molecular markers should be investigated.

Finally, the knowledge of enzymatic and non-enzymatic oxidative defense mechanisms will serve as a guiding principle for establishing the most effective nutrition intake to ensure adequate biological support, especially in patients affected by liver diseases. Investigations into the relationship between the dietary intake and OS mediators, which are enhance the inflammatory response directly and/or indirectly, are needed.

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REVIEW

Emerging concepts in liver graft preservation

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Abstract

The urgent need to expand the donor pool in order to attend to the growing demand for liver transplantation has obliged physicians to consider the use of suboptimal liver grafts and also to redefine the preservation strategies. This review examines the different methods of liver graft preservation, focusing on the latest advances in both static cold storage and machine perfusion (MP). The new strategies for static cold storage are mainly designed to increase the fatty liver graft preservation *via* the supplementation of commercial organ preservation solutions with additives. In this paper we stress the importance of carrying out effective graft washout after static cold preservation, and present a detailed discussion of the future perspectives for dynamic graft preservation using MP at different temperatures (hypothermia at 4 ℃, normothermia at 37 ℃ and subnormothermia at 20 ℃-25 ℃). Finally, we highlight some emerging applications of regenerative medicine in liver graft preservation. In conclusion, this review discusses the "state of the art" and future perspectives in static and dynamic liver graft preservation in order to improve graft viability.

Key words: Static cold preservation; Suboptimal liver grafts; Preservation solutions; Graft washout solutions; Machine perfusion and liver bioengineering

Core tip: This review focuses on the latest advances in liver graft preservation, in both static cold storage and dynamic preservation by machine perfusion (MP). We describe some new trends for static cold preservation based on our experience; we stress the importance of developing washout solutions and the use of MP for suboptimal liver grafts. Finally, we discuss emerging applications of regenerative medicine in liver graft preservation.

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INTRODUCTION

Liver transplantation is the definitive treatment option for end-stage liver diseases. Besides the immunological mechanisms of graft rejection, liver transplantation outcome is also limited by ischemia-reperfusion injury (IRI). IRI is a complex multifactorial process caused, principally, by the energy depletion during graft cold storage in preservation solutions (cold ischemia) and the subsequent production of oxidative stress and inflammatory events after graft revascularization in the recipient (reperfusion)[1]. IRI is associated with delayed graft function and primary graft failure, which remains one of the major clinical problems following liver transplantation.

A common strategy to reduce ischemic injury following explantation from the donor is the rapid cooling of the organs with the use of a preservation solution to minimize enzymatic activity and energy substrate depletion. In recent decades, major advances have been made in the area of liver preservation, including the development of new preservation solutions. Their emergence has helped to decrease hypoxic injury and has reduced graft vulnerability against reperfusion insult.

Currently, the high increase in demand for organs has obliged physicians to use suboptimal grafts in order to increase the organ supply for transplantation. Suboptimal or extended criteria donor (ECD) livers include organs characterized by steatosis, old donor age, prolonged cold ischemia or donation after cardiac death (DCD)[2,3]. It is well known that suboptimal livers present increased vulnerability to IRI, and are associated with graft dysfunction and long-term survival problems after surgery. For this reason, preservation methods for suboptimal livers need to be exhaustively explored in order to identify the ones that are the most suitable for graft conservation.

Machine perfusion (MP) has emerged as an alternative preservation strategy to static cold storage (SCS). MP is already routinely used for kidney transplantation, but a great deal is still to be done before it can be regularly used in clinical liver transplantation. In this review, we examine the SCS and MP techniques in detail, describing the latest advances in the development of preservation solutions for liver grafts and providing some proposals and new strategies in order to improve current graft preservation methods.

STATIC COLD STORAGE

The main goal in organ preservation is to maintain function of the organ and tissue during storage so that the graft will be viable at reperfusion. To date, the predominant organ preservation method used by most centers is SCS. The principles of SCS are based on the diminution of metabolism by hypothermia. The appropriate preservation solution is infused into the organ (the cooling phase) and then stored statically[4].

Cooling

SCS is the most widely used method for preserving organs for transplantation. Cooling is necessary to reduce cellular metabolism and the oxygen requirements in order to prevent tissue injury[5].

In order to obtain viable organs after long-term preservation, various methods have been proposed, ranging from organ freezing and vitrification[6,7] to "supercooling" (subzero non-freezing at 0 ℃ to -5 ℃)[8-11]. In general, long-term survival rates after transplantaion using these methods are disappointing.

However, in a recent study by Berendsen *et al*[12], the combination of "supercooling" (cold preservation at -6 ℃) with other parameters achieved effective preservation of liver grafts for 4 d. This promising new technique comprises three steps: first, "supercooling" of the organ at -6 ℃ to reduce the cellular metabolism; second, subnormothermic MP at 21 ℃ (see the dynamic preservation section below), which reinitiates the metabolism and replenishes ATP levels, and third, the use of two preservatives, 3-O-methyl-D-glucose (3-OMG) and polyethylene-glycol 35 (PEG35). Each of these conditions is necessary to achieve successful liver transplantation[13]. With this in mind, supercooling techniques may be a potentially useful tool for suboptimal livers which are currently discarded for transplantation purposes, and may have great impact on global organ sharing.

Preservation solutions

Although cold is a fundamental requirement for tissue preservation, it has harmful repercussions due to the induction of cell swelling[14] and cytoskeletal alteration[15]. This was in part the reason for the development of commercial organ preservation solutions able to prevent many of the cellular alterations associated with hypothermia and to mitigate the harmful effects of cooling.

Euro-Collins (EC) solution was developed in the 1970s as a high potassium-sodium solution (intracellular composition) which does not contain oncotic agents but does contain glucose. Given that glucose is impermeable to renal cells, this preservation solution was suitable for kidney preservation when relatively short times were needed or DCD organs were used. However, the permeability of the liver and pancreatic cells to glucose leads to the loss of the osmotic effect, and also causes the subsequent anaerobic metabolization of glucose, inducing intracellular acidosis and thus limiting cell preservation. This is why glucose was later substituted by other larger sugar molecules such as lactobionate and raffinose in

Table 1 Additives for improving static cold storage in University of Wisconsin and Institute Georges Lopez preservation solutions

Additive	Preservation solution	Ref.
TMZ	UW, IGL-1	[25,30]
EGF + IGF-1	UW	[45]
IGF-1	IGL-1	[44]
EGF	IGL-1	[46]
ML	IGL-1	[43]
BZ	UW, IGL-1	[51,117]
SV	UW	[64]
BZ, MG132	UW	[50]
ML + TMZ	IGL-1	[38]
CAII	IGL-1	[54]

TMZ: Trimetazidine; EGF: Endothelial growth factor; IGF: Insulin growth factor 1; ML: Melatonin; BZ: Bortezomib; SV: Simvastatin; CAII: Carbonic anhydrase Ⅱ; UW: University of Wisconsin; IGL-1: Institute Georges Lopez.

University of Wisconsin (UW) solution, which remains in the extracellular space and preserves its beneficial effect. The use of the UW preservation solution improved organ preservation time from 6 to 16 h[16].

The efficacy of UW solution is based on the prevention of edema by impermeants (raffinose, lactobionate), and the addition of an ATP precursor (adenosine) and anti-oxidant components (allopurinol, reduced glutathione). Drawbacks include the presence of hydroxyethyl starch (HES) as oncotic support, which has been associated with high blood viscosity and consequent tissue saturation with the preservation solution. As a result, washout of blood from the graft and blood flow during reperfusion may be reduced[17,18]. In addition, the high K^+ concentration is associated with cellular depolarization and activation of voltage-dependent channels[19]. The problems caused by HES and K^+ led to the development of other preservation solutions without oncotic agents such as Celsior and HTK (Custodiol) and others with PEG as oncotic agent, such as Institute Georges Lopez solution (IGL-1) and Tissue and Organ Conservation Solution (SCOT).

Celsior was developed initially in the 1990s as a cardiac preservation solution with a low potassium and high sodium composition. Due to its extracellular composition, Celsior was also adopted for the preservation of abdominal organs as an alternative to UW. Other solutions without oncotic agents such as histidine-tryptophan-ketoglutarate solution (HTK) were also developed. HTK presents low viscosity and for this reason provides more rapid cooling and better washout of blood elements during organ procurement than UW. Celsior and HTK solutions have been extensively used for liver transplantation[20-22]. However, some limitations for HTK use have recently been described. Stewart *et al*[23] reported that HTK is associated with reduced graft survival in case of additional risk factors such as DCD, cold ischemia time over 8 h, and donors over 70 years when compared to UW solution.

In IGL-1 preservation solution, HES was substituted by a PEG with a molecular weight of 35 KDa (PEG35), and the high K^+/low Na^+ ratio was reversed. Both experimental[24,25] and clinical[26-28] studies of liver and kidney transplantation have shown the beneficial effects of IGL-1 against apoptosis, endoplasmic reticulum stress, microcirculation dysfunction and immune response. Moreover, in previous studies of cold preservation and *ex vivo* perfusion, we have reported that IGL-1 contributes to a more efficient preservation of both non-steatotic and steatotic rat liver grafts compared to UW[29-31], The beneficial effects of IGL-1 include prevention of hepatic damage, oxidative stress and mitochondrial injury, and are mediated through nitric oxide (NO) production. So IGL-1 is the first solution reported to be advantageous in SCS of suboptimal livers.

Moreover, a PEG of smaller size, PEG20, is the basic component of another solution for organ preservation: the SCOT, which furthermore contains low K^+/high Na^+ concentrations. SCOT was reported to show a higher renal protection against the immune response, mainly due to the "immunocamouflage" process provided by PEG20[32]. PEG20 at 15 g/L has been found to reduce alloantigen recognition after liver reperfusion in comparison to UW solution[33]. Even so, the use of PEG35 as oncotic agent has been shown to be more effective than PEG20 for liver graft preservation[34].

Modification of static preservation solutions

The extended use of commercial preservation solutions has improved the conditions of liver graft preservation, but with the increasing use of suboptimal grafts it seems necessary to explore new alternatives in order to prolong the ischemia times and increase graft quality during cold storage. Along these lines, new additives have been proposed to improve static liver graft preservation when UW and IGL-1 solutions are used (Table 1). Although these alternatives are promising and have been successfully applied in animal models, they require further investigation before they can be implemented in clinical transplantation.

Anti-ischemic drugs: Previous work in kidney[35,36], liver[37,38] and heart[39-41] models has demonstrated the anti-oxidant action of trimetazidine (TMZ), an anti-ischemic drug. The addition of TMZ to UW solution was tested in both steatotic and non-steatotic rat livers after cold storage and *ex vivo* perfusion[25]. The enrichment of UW solution with TMZ reduced hepatic injury by diminishing microcirculatory dysfunction, oxidative stress, and mitochondrial damage. In the same experimental conditions, supplementation of IGL-1 solution with TMZ offered better liver graft preservation than IGL-1 solution alone and induced significant activation of hypoxia inducible factor-1α (HIF1α) and increased NO production[30]. The benefits of TMZ have been shown clinically in patients undergoing hepatic surgery under vascular clamping[42]. This would suggest that TMZ has

potential for use as an additive in commercial preservation solutions for clinical transplantation purposes.

Hormones: Melatonin (ML), a hormone produced by the pineal gland in a circadian manner, has been shown to be highly beneficial for enhancing resistance of both steatotic and non-steatotic livers against IRI when added to IGL-1. ML decreased hepatic injury by overexpression of endothelial NO synthase (e-NOS) and Heme Oxygenase-1, and reduced mitochondrial damage and oxidative stress[43]. These protective effects of ML in fatty liver graft preservation were further potentiated by addition of TMZ to IGL-1 + ML solution[38]. Protective mechanisms were dependent on AMPK activation. Furthermore, UW and IGL-1 solutions enriched with trophic factors like epidermal growth factor and insulin-like growth factor-1 enhanced the resistance of steatotic livers to IRI, partly due to Akt and eNOS signaling activation, and reduced cytokine release[44-46].

Proteasome inhibitors: The ubiquitin proteasome system (UPS) is an energy-dependent system that degrades misfolded proteins and regulates various cellular processes[47]. It has been established that proteasome activation is a pathophysiologically relevant mechanism of cold ischemic myocardial injury. A subset of 26S proteasomes appears to be a cell-destructive protease that is activated as ATP levels decline[48]. The addition to UW solution of epoxomicin, a proteasome inhibitor, reduced cardiac edema and preserved the ultrastructural integrity of the post-ischemic cardiomyocyte[49]. In liver, we have recently demonstrated that the addition of the reversible UPS inhibitors bortezomib (BRZ) and MG132 to UW solution improved steatotic and non-steatotic liver preservation, and that the protective effect of BRZ was superior to that of MG132[50]. Supplementation of IGL-1 solution with BRZ also showed protective effects which were partially mediated through the activation of AMPK and Akt/mTOR signaling[51].

Carbonic anhydrase Ⅱ: Carbonic anhydrase (CA) are Zn-metalloenzymes that catalyze the reversible reaction between carbon dioxide hydration and bicarbonate dehydration. Recently the function of CAs has aroused great interest, as they contribute to the transport of CO_2 and protons across the biological membranes and are involved in pH regulation, CO_2 homeostasis and biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis. In mammals 16 different CAs are found, with different amino acid sequences, enzymatic properties and sites of expression[52]. Since carbonic anhydrase Ⅱ (CAⅡ) also contributes to acid-base homeostasis[53], we suggest that it could be modulated in conditions of liver preservation and that its addition to the preservation solution could be an efficient strategy for reversing pH alterations provoked by cold ischemia. Indeed, in preliminary studies at our laboratory, we have observed that fatty livers preserved in IGL-1 solution supplemented with CAⅡ showed lower injury, better function and major reductions in liver apoptosis parameters[54]. So CA enrichment of preservation solutions is an up-and-coming approach for improving the preservation of suboptimal liver grafts.

Statins: Statins, or the 3-hydroxyl-3-methylglutaryl coenzyme A inhibitor family, are a group of drugs known to decrease cholesterol levels and treat dyslipidemias[55]. They also have a variety of anti-inflammatory, antioxidant and immunoregulatory effects[56,57] and they maintain the endothelial barrier by activation of eNOS and subsequent production of NO[58-60]. Due to their various effects, statins have been proposed as effective pharmacological agents against IRI in both normal and steatotic livers[61-63]. UW supplementation with simvastatin (a synthetic analog of statin) prevented the deleterious effects of cold storage in endothelial cells, due to the enhancement of vasoprotective pathways, thus improving liver viability[64]. With this in mind, the supplementation of IGL-1 with simvastatin could promote the NO generation induced by IGL-1 solution alone, and may contribute to preventing the exacerbated microcirculation complications existing in fatty liver grafts after revascularization. In addition, increased levels of NO could contribute to stabilize cytoprotective factors such as HIF-α, which are generated as an adaptive response to the hypoxic conditions that characterize cold preservation[30].

New potential additives: Some considerations

Sirtuin activators: Sirtuin1 (SIRT1) is a deacetylase that regulates the activity of various non-histone and histone proteins and as a result is involved in various cell processes such as apoptosis and oxidative stress[65-68]. SIRT1 induces AMPK activation through LKB1 deacetylation, and favors NO production by e-NOS activation[69,70]. Further, in a recent study published by our group, we mentioned that SIRT1 is involved in the beneficial effects of ischemic preconditioning, partly *via* AMPK and eNOS activation[68]. Consequently, addition of SIRT1 activators in preservation solutions may be a promising strategy for prolonging storage periods; SIRT1 activators may activate AMPK and maintain the cell energy status, and may also increase NO levels and alleviate microcirculation disturbances, especially in fatty livers. Preliminary data obtained from our laboratory showed that SIRT1 is a differential marker in steatotic and non-steatotic livers during cold preservation. Since SIRT1 activity requires high NAD^+ levels, NAD^+ activators may also contribute to better liver graft preservation by activating not only SIRT1, but also other members of sirtuin-family such as Sirtuin3 (SIRT3). SIRT3 is located in the mitochondria and affects the acetylation status of various mitochondrial proteins[71]. Enhancement of SIRT3 activity could thus achieve better mitochondrial preservation and prevent reactive oxygen

species (ROS) production during reperfusion.

Nrf2 activators: Moreover, recent studies have demonstrated the importance of Nrf2 in IRI models[72-74]. Nrf2 is activated under conditions of oxidative stress and induces the transcription of anti-oxidant enzymes in order to eliminate redox stress. Nrf2-deficient livers exhibit enhanced liver injury upon IRI[75]. Consequently, we propose the use of Nrf2 activators in preservation solutions in order to alleviate oxidative stress during reperfusion.

All in all, extensive studies in experimental models have proposed modified preservation solutions in order to extend cold storage and to maintain graft viability as far as possible. Since IRI is a multifactorial process, preservation solutions could incorporate various pharmacological agents in order to combine diffe-rent protective mechanisms and thus improve liver preservation. Nonetheless, the use of pharmacological agents may be limited by their potential toxicity and side effects or their unsuitability for suboptimal grafts, and so novel strategies of preservation should be developed.

Liver graft wash out

After cold storage, the liver grafts preserved in commercial preservation solutions need to be washed out to remove the solution before reperfusion and also to obtain the most suitable conditions for graft revascularization and viability after transplantation. Although research into rinse solutions is limited, recent data from our laboratory show that washing out the liver grafts preserved in UW for 24 h, with a rinse solution containing PEG35, is an effective tool for reducing liver graft injury after two hours of *ex vivo* perfusion[76]. PEG35 in the rinse solution was associated with decreased oxidative stress and mitochondrial damage, increased activation of AMPK, and enhanced NO generation. In addition, it contributed to restoring cytoskeleton integrity following IRI. In contrast, when livers were preserved in IGL-1 solution, these benefits were not evident, probably due to the presence of PEG35 as oncotic agent (unpublished data).

It is well known that PEG molecules are water-soluble polymers of various molecular weights which are non-immunogenic and non-toxic[77]. In general, PEGs prevent the generation of ROS[78,79], enhance cell survival pathways in hypoxia/reoxygenation conditions and repair endothelial cell damage during post-ischemic reperfusion[80,81]. PEG exerts its cytoprotective role through the restoration of membrane integrity[15,78,81,82] or by entering the cell through the disrupted membranes and interacting with cellular organelles[83]. In hypothermic hepatocyte preservation, PEG8 (8 kDa) prevented cell swelling through a mechanism that was independent of its osmotic properties[14].

DYNAMIC PRESERVATION: MACHINE PERFUSION TECHNIQUES

For standard liver grafts, SCS with different preservation solutions remains highly successful. However, with the increasing need for organs in recent years, the use of novel techniques for optimizing suboptimal graft preservation is arousing interest.

MP consists of creating a controlled recirculating flow of preservation solution through the organ using a pump. This continuous perfusion permits better penetration of the preservation solution, a thorough washout of blood and equilibration of the interstitium with the perfusate medium, delivery of oxygen and nutrients (if the perfusate is oxygenated), and removal of toxic metabolites (when the perfusate is renewed or filtered). In addition, it allows real-time monitoring of the functional and biochemical performance of the graft and the provision of metabolic support during preservation[84].

Unlike the kidney, the MP protocol for the liver is determined mainly by the temperature of preservation: hypothermic (HMP) at 4 ℃, normothermic (NMP) at 37 ℃ and subnormothermic (SNMP) at 20 ℃-25 ℃. Also, several flows and pressures (pulsatile or not), single or dual perfusion (hepatic artery and portal vein), oxygenation or non-oxygenation, and different MP solution compositions have been tested in various liver graft experimental models[85].

HMP

HMP is a dynamic cold preservation method at 4 ℃ which ensures homogeneous and continuous supply of metabolic substrates to the graft during the *ex vivo* period[86]. During HMP, aerobic metabolism decreases but does not stop completely and the provision of metabolic substrates allows the reduction of the cellular insults seen during reperfusion.

HMP offers several advantages over SCS. Guarrera *et al*[87] were the first to compare HMP to SCS in human liver transplantation, and showed that HMP improves graft function and attenuates classical biochemical markers of liver preservation injury. Given the fact that ROS accumulation during ischemia can lead to significant hepatocyte toxicity, HMP has been shown to protect the rodent liver from ROS by a reduction in glutathione depletion and superoxide anion release when compared with SCS[88]. And in the case of suboptimal livers, Bessems *et al*[89] showed that HMP improved both hepatocellular and endothelial function while reducing damage in a diet-induced rat fatty liver model.

In contrast to the kidney, in which successful HMP does not necessarily depend upon oxygenation, oxygenated HMP (HOPE) has been developed as a means of improving the quality of liver preservation in normal

or ECD livers[90]. Oxygenated preservation enables grafts to restore tissue homeostasis and to maintain the functional integrity of hepatocytes during ischemia. In a recent study, Schlegel *et al*[91] also described a protective effect on the rodent biliary system using HOPE in DCD grafts that underwent transplantation. As expected, perfusion with the HOPE system decreased the parameters of hepatocellular injury and lowered immunogenic upregulation.

Perfusates for HMP: In general, the composition of perfusate solutions used for HMP is based on a re-formulation of UW solution, in which lactobionate is replaced by gluconate. This solution, named Belzer-MP solution (Belzer-MPS), continues to be the predominant perfusion solution.

Bessems *et al*[92] described a new HMP solution, Polysol, which contains amino acids, histidine, glutamine, tryptophan, ascorbic acid and α-tocopherol. Their studies show that Polysol improved liver preservation compared to Belzer's MPS, with lower enzyme release and increased bile production. Vasosol has also been proposed as an efficient alternative for HMP[87]. Its composition is based on Belzer-MPS but it is supplemented with antioxidants (N-acetyl-cysteine), metabolic substrates (α-ketoglutarate, *L*-arginine) and vasodilators (prostaglandin E1 and nitroglycerin). Recently, the benefits of Vasosol have been improved by the addition of α-tocopherol to further enhance antioxidant properties when HMP is used[93].

SNMP

Recently it has been suggested that the use of SNMP systems may be suitable for *ex vivo* preservation and recovery of human liver for transplantation. SNMP is an intermediate status for graft conservation, using sub-thermic conditions (20 ℃-25 ℃), taking advantage of the lower metabolic demand in sub-physiological temperature conditions, while still maintaining sufficient metabolism for viability testing and improvement of graft function. SNMP has already proven advantageous in reducing markers of biliary injury during preservation and in restoring normal biliary physiology[94]. A recent study by Bruinsma *et al*[95] is the first demonstration of the capacity of SNMP to sustain human livers. This group showed that SNMP effectively supports the human liver *ex vivo* with minimal injury, and normalizes physiological post-ischemia disturbances.

NMP

The principle of normothermic perfusion is the maintenance of normal cellular metabolism in a physio-logical environment throughout the preservation period by maintaining normal temperature (37 ℃) and providing oxygen and essential substrates[96]. This ensures large-scale metabolic activity and the maintenance of energy reserves such as ATP content. NMP has the advantage of allowing viability assessment prior to transplantation. As the liver metabolism is maintained during preservation, markers including bile production and liver enzymes can be measured.

NMP is an emerging technology whose potential in liver preservation has been described in several animal studies, which have shown its superiority over SCS in the preservation of liver grafts[96-98]. Interestingly, porcine and murine models of DCD livers are significantly improved by NMP compared to organs preserved by SCS[99,100].

Recently, Ravikumar *et al*[101] reported the first clinical trial of transplanted livers with NMP. Their study included 10 transplanted patients with relatively low risk donors and recipients, and showed that NMP is safe and feasible in human applications. This study opens up new avenues for research into liver graft preservation with NMP.

Recently, NMP has emerged as a novel tool for decreasing steatosis in a process named "defatting". In a preliminary study using porcine livers, *ex vivo* normothermic perfusion for 48 h led to a 50% reduction in lipid droplet size in perivenous hepatocytes, reaching the size found in control lean livers[102]. Moreover, NMP of steatotic livers from Zucker ob rats using a "defatting cocktail" decreased the intracellular lipid content by 50% over 3 h of perfusion[103]. Decreasing steatosis prior to transplantation by short term NMP would allow the transplantation of severely steatotic livers and thus alleviate the donor liver shortage.

Perfusates for NMP: NMP requires advanced metabolic support since the organ is fully metabolically active. Therefore, typically diluted blood-based perfusates are used. More recently, a solution initially described for lung perfusion has also been applied to liver grafts[104]. Steen is a buffered extracellular solution containing dextran and albumin at an optimized colloid osmotic pressure.

For defatting purposes, the perfusate developed contains different compounds to activate nuclear receptors such as PPARs, pregnane X receptor, and constitutive androstane receptor in order to exert an insulin-mimetic effect and to stimulate intracellular cAMP. This liquid was added into Minimum Essential cell culture medium as a perfusate to stimulate the lipid metabolism of obese rat liver grafts preserved using NMP. With this cocktail, a significant decrease (50%) in steatosis was observed after 3 h of NMP[103]. A recent study showed that the supplementation of this cocktail with *L*-carnitine, together with hyperoxic exposure, abolished the sensitivity of macrosteatotic hepatocytes to hypoxia reoxygenation (H/R)[105].

BIOENGINEERING IN LIVER GRAFT PRESERVATION

In the context of liver graft preservation, bioengineered human livers represent an opportunity to test new solutions and liver preservation methods, thus potentially bypassing the requirement of precious and scarce human organs. Bioengineering allows quicker and cheaper

Table 2 Advantages and disadvantages of machine perfusion preservation

Advantages	Disadvantages
Continuous nutrients and oxygen supply	Logistically complex
Continuous monitoring of organ viability	High cost
Removal of metabolic waste products	No optimized conditions
Extended preservation time	Need for trained personnel
Better preservation of microcirculation	
Potential "rescue" of suboptimal organs	

development and transfer to the clinic[106].

Over the past few years, organ bioengineering has come of age. The seminal study by Ott *et al*[107] in 2008 on heart decellularization and recellularization paved the way for whole organ bioengineering. After this initial study of the heart, many other organs followed. In 2009, Baptista *et al*[108] described the first methods for liver, pancreas and kidney decellularization and recellularization, and their paper was followed by an exponential growth of publications by many other authors.

Currently, with several solid organs already successfully bioengineered and under further development by several groups around the world, this technology has huge potential. However, bioengineered organs are still not available to the transplant surgeon as alternative grafts. There are already several applications that can be addressed and extended with the current generation of bioengineered organs and their acellular scaffolds. Most of these applications, like drug metabolism[106], organ/tissue physiology[106,107,109,110], matrix biology[111], developmental biology[111,112], and stem cell biology[113] are perfectly complemented by these novel bioengineered human tissues which will open up exciting new experimental avenues.

In the particular context of normothermic perfusion, the enabling bioreactor and culture media technology developed in the bioengineering process of livers may constitute a new body of knowledge that can help further the development of NMP for liver preservation, due to the similarities of the conditions used[114]. Finally, the use of normothermic perfusion bioreactors in liver preservation and bioengineered human livers may also provide a better route and environment for *ex vivo* administration of mesenchymal stem cells. The use of these cells has been proposed as a novel way to attenuate IRI and to downregulate the alloimmune response (adaptive immunity) and promotes engraftment after transplantation[115]. This has been demonstrated for rat kidneys, thus raising the hope that it may also work in the liver and other solid organs[116].

CONCLUSION

Due to its low cost and simple technical and logistical requirements, SCS is still preferred to MP as the standard method of preservation in liver transplantation. SCS is probably unsuitable for suboptimal liver grafts, because they have already suffered severe tissue damage secondary to hypoxia during the initial period of warm ischemia. Additional damage to the organ due to hypothermic conditions may limit the ability to restore cellular function, because metabolic activity is decreased at low temperatures.

The growing need to use suboptimal livers and to expand donor pool is accompanied by the drive to improve current preservation techniques before transplantation. In this situation, there has been renewed interest in liver graft preservation using machine perfusion. Both HMP and NMP have been found to be beneficial in preserving normal and suboptimal livers, and their relative merits are currently being debated. More basic research and randomized controlled trials are needed. As for SNMP preservation, it remains relatively unexplored at present.

Studies on the cost-effectiveness of MP and SCS will continue over the coming years, but considerable support for MP is beginning to emerge. Table 2 summarizes its advantages and disadvantages for liver preservation. It seems clear that MP strategies will play an increasing role and that their use should be optimized, including the subsequent development of new perfusion solutions. With this in mind, the future of liver MP preservation will also depend on the composition of perfusion solutions. At present, little attention is being paid to the potential advantages of adding cytoprotective, immunomodulating, pro-regenerative components to the MP solutions.

It is well known that PEG protects cell membranes; it has already been used as a colloid in machine perfusion, just as it was previously in SCS. The development of different PEG molecules could establish new frontiers in the design of new perfusion solutions for application in MP techniques and may increase graft conservation in the future. The revitalization of steatotic livers through defatting agents represents another interesting future application, given that the worldwide incidence of severely steatotic livers is expected to rise together with the increase in obesity rates.

Finally, bioengineering is another area with great potential for graft preservation in clinical transplantation.

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REVIEW

Management of hepatitis C in patients with chronic kidney disease

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Abstract

Hepatitis C virus (HCV) infection is highly prevalent among chronic kidney disease (CKD) subjects under hemodialysis and in kidney transplantation (KT) recipients, being an important cause of morbidity and mortality in these patients. The vast majority of HCV chronic infections in the hemodialysis setting are currently attributable to nosocomial transmission. Acute and chronic hepatitis C exhibits distinct clinical and laboratorial features, which can impact on management and treatment decisions. In hemodialysis subjects, acute infections are usually asymptomatic and anicteric; since spontaneous viral clearance is very uncommon in this context, acute infections should be treated as soon as possible. In KT recipients, the occurrence of acute hepatitis C can have a more severe course, with a rapid progression of liver fibrosis. In these patients, it is recommended to use pegylated interferon (PEG-IFN) in combination with ribavirin, with doses adjusted according to estimated glomerular filtration rate. There is no evidence suggesting that chronic hepatitis C exhibits a more aggressive course in CKD subjects under conservative management. In these subjects, indication of treatment with PEG-IFN plus ribavirin relies on the CKD stage, rate of progression of renal dysfunction and the possibility of a preemptive transplant. HCV infection has been associated with both liver disease-related deaths and cardiovascular mortality in hemodialysis patients. Among those individuals, low HCV viral loads and the phenomenon of intermittent HCV viremia are often observed, and sequential HCV RNA monitoring is needed. Despite the poor tolerability and suboptimal efficacy of antiviral therapy in CKD patients, many patients can achieve sustained virological response, which improve patient and graft outcomes. Hepatitis C eradication before KT theoretically improves survival and reduces the occurrence of chronic graft nephropathy, *de novo* glomerulonephritis and post-transplant diabetes mellitus.

Key words: Hepatitis C virus; Chronic kidney disease; End-stage renal disease; Hemodialysis; Kidney transplantation; Diagnosis; Conservative management; Therapy

Core tip: In this review, we discuss the most recent and relevant literature regarding diagnostic aspects, clinical features, outcomes and therapy of chronic hepatitis C

in subjects with chronic kidney disease, in the context of conservative management, hemodialysis, and kidney transplantation. In addition, antiviral regimens are summarized and treatment algorithms are proposed.

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INTRODUCTION

Over the last two decades, there has been a large body of evidence that supports an intimate relationship between liver and kidney diseases. In the same way that several causes of renal injury can occur in patients with acute liver failure or chronic liver disease, a variety of hepatic lesions can develop in subjects with chronic kidney disease (CKD). Although drug-induced liver injuries, non-alcoholic fatty liver disease and hepatic iron overload are relatively frequent in CKD patients, hepatitis C virus (HCV) infection remains the most common and severe cause of liver disease in this population[1].

HCV infection is a major public health issue, which affects approximately 2.8% of the world's population[2,3]. HCV infection is highly prevalent among CKD subjects and, consequently, in kidney transplant (KT) recipients[4,5]. In spite of the reduction in HCV seroconversion rates in hemodialysis units, prevalence is still substantially higher than in general population, ranging from 10% to as high as 59%, according to the geographic area[6,7]. A recent meta-analysis performed by Su *et al*[8] on the incidence of HCV infection in hemodialysis patients confirmed this high variability of incidence rates across regions, with most of this heterogeneity probably related to the level of country development and differences in the primary prevalence of HCV infection in hemodialysis units. By evaluating 22 studies, these authors found a pooled incidence rate of HCV infection of 0.97 (95%CI: 0.66-1.29) in developed countries, and of 4.44 (95%CI: 2.65-6.23) per 100 patients in developing countries[8]. Patients under renal replacement therapy, particularly hemodialysis, are exposed to blood borne pathogens, given the need for intravenous access, and frequent catheter manipulation[9]. These patients are frequently treated in close proximity to one another and share supplies or equipment that can become contaminated. Furthermore, breaches in infection control practices can result in episodes of patient-to-patient HCV transmission[10]. Time in hemodialysis, previous renal transplant and presence of anti-HBc antibodies are associated with HCV infection while use of erythropoietin (EPO) and adherence to universal precaution measures seem to protect against HCV infection[11]. While transfusion of blood products still plays a significant epidemiological role in developing countries, the vast majority of HCV chronic infections in the hemodialysis setting are currently attributable to nosocomial transmission through hand-borne transmission or by the use of contaminated medication vials, such as saline, anesthetic drugs and unfractionated heparin (UFH)[6,9,12]. Although single dose low molecular weight heparin (LMWH) has been increasingly used (particularly in Western Europe), UFH provided in multi-dose vials is the anticoagulant of choice for most maintenance hemodialysis units all over the world, which possibly contributes to HCV transmission when standard precautions are not strictly adopted[13,14].

Since there is a paucity of data for individuals on peritoneal dialysis, this review will focus on diagnostic aspects, clinical outcomes and therapeutic options for hepatitis C in CKD patients receiving conservative management, undergoing hemodialysis, and after kidney transplantation.

ACUTE HEPATITIS C

With the introduction of EPO and the consequent reduction of blood transfusions in hemodialysis patients, the main route of HCV infection is related to environmental transmission of the virus[15,16].

In hemodialysis patients, acute infections are usually asymptomatic and anicteric. Despite the lower levels of alanine aminotransferase (ALT) levels observed in CKD patients[17,18], acute infections are often accompanied of moderate ALT elevations (typically inferior to 10 times the upper limit of normality), followed by anti-HCV seroconversion in 90% of cases, one to seven months after ALT elevation[16,19-21]. Systematic screening of ALT and anti-HCV in hemodialysis patients are strongly recommended (monthly for ALT and 6-monthly for anti-HCV), and even small unexplained increase in serum ALT levels should raise the suspicion of acute HCV infection. The infection is confirmed by the detection of HCV RNA in serum by polymerase chain reaction (PCR) assay, which precedes the appearance of anti-HCV antibodies by several weeks or months[22,23]. In the study of Moreira *et al*[24], serum samples were collected monthly for 1 year from 281 patients admitted for hemodialysis; six patients seroconverted during the study (incidence = 3.1/1000 person-month). In 1.8% (5/281) of cases, RNA was detected before the appearance of antibodies (up to 5 mo), and in 1.1% (3/281) of cases, RNA was the unique marker of HCV infection.

Viral clearance is very uncommon in hemodialysis patients, occurring in less than 5% of patients[16,19], and therefore acute infections should be treated as soon as the diagnosis is established, whenever possible. Given that documentation of anti-HCV seroconversion is generally feasible in the context of hemodialysis, a pre-treatment liver biopsy is seldom necessary, unless a differential diagnosis is required.

In non-uremic patients, data about treatment of

Table 1 Studies on acute hepatitis C treatment in hemodialysis patients

Ref.	Süleymanlar *et al*[26] (1998)	Gürsoy *et al*[27] (2001)	Urbánek *et al*[28] (2004)	Al-Harbi *et al*[29] (2005)	Rocha *et al*[30] (2007)	Engel *et al*[32] (2007)	Liu *et al*[33] (2010)	Ferreira *et al*[31] (2011)
n	3	36	18	9	23	10	35	26
Interferon type	IFN	IFN	IFN	IFN	IFN	PEG-IFN α2b	PEG-IFN α2a	IFN PEG-IFN α2a
Schedule	4.5 MU *tiw*	3 MU or 6-10 MU *tiw*	10 MU + 3 MU *tiw*	3 MU *tiw*	3 MU or 6 MU *tiw*	1 μg/kg *qw*	135 μgv *qw*	3 MU *tiw* or 135 μg *qw*
Duration	16 wk	12 wk	3 wk + 12 wk	12 wk	48 or 24 wk	24 wk	24 wk	48 wk
SVR	100%	39%	72%	67%	43%	40%	89%	54%

IFN: Interferon; PEG-IFN: Pegylated interferon; MU: Million units; *tiw*: Three times a week; *qw*: Once a week; SVR: Sustained virological response rate by intention-to-treat analysis.

Table 2 Treatment regimens for hepatitis C virus infection in chronic kidney patients

Stage of CKD	Estimated GFR	Target dosage of ribavirin	Dosage of Interferon
1	⩾ 90	800 to 1200 mg *qd*[1]	PEG-IFN α2a 180 μg *qw*
2	60 to 89	600 to 800 mg *qd*[1]	or PEG-IFN α2b 1.5 μg/kg *qw*
3	30 to 59	400 to 600 mg *qd*[1]	
4	15 to 29	200 mg *qd*	PEG-IFN α2a 135 μg *qw*
5	< 15 or HD	Titrated according to patient tolerability[2]	or PEG-IFN α2b 1.0 μg/kg *qw*

[1]Divided in two doses; [2]See text for details. GFR: Glomerular filtration rate expressed in mL/min per 1.73 m^2; *qd*: Once a day; PEG-IFN: Pegylated interferon; *qw*: Once a week; HD: Hemodyalisis.

acute hepatitis C are limited and heterogeneous regarding studied populations, regimens and duration of treatment[25]. In hemodialysis patients data are even scarcer, with small sample sizes. In addition, most studies report results with standard interferon[26-31]. Only two studies reported data of pegylated interferon (PEG-IFN) in hemodialysis patients[32,33]. These two and six other studies were evaluated in a meta-analysis about treatment of acute hepatitis C in hemodialysis patients[34] (Table 1). The global rate of sustained virological response (SVR) was 59%, with 9% of dropouts. Although there were no clear differences in efficacy or safety between PEG-IFN and standard IFN, we suggest that PEG-IFN with adjusted doses (Table 2) should be preferentially used, for the sake of better patient compliance and comfort. In non-uremic patients there is no evidence of additional benefit of association with ribavirin[35] but there is no data regarding its use in uremic patients. Nevertheless, the recommendation is to treat HCV acute infection with monotherapy PEG-IFN for six months, regardless of the genotype. It is not recommended to wait 12 wk for spontaneous clearance, since this occurrence is very uncommon in CKD subjects.

In KT recipients, the occurrence of acute hepatitis C can be associated with a more severe course, with a rapid progression of fibrosis towards cirrhosis, including the development of fibrosing cholestatic hepatitis or vanishing bile duct syndrome[36-39]. For this reason, antiviral therapy should be rapidly introduced, even if poor tolerability and efficacy are expected. Although there are no comparative studies with IFN monotherapy, the better option would be the treatment with PEG-IFN in combination with ribavirin for 24 to 48 wk, with doses adjusted according to estimated glomerular filtration rate (eGFR) (Table 2).

CHRONIC HEPATITIS C BEFORE KIDNEY TRANSPLANTATION

CKD patients under conservative management

The prevalence of HCV infection is higher in conservative management CKD patients than in general population, being mainly related to parenteral exposure[40-44]. Clinical and laboratory features of chronic HCV infection in CKD individuals under conservative management are not well known, and additional studies are needed to better understand the natural history and clinical impact of chronic HCV infection in this population. Nevertheless, the accuracy of ALT in detecting HCV infection is high[43,44], suggesting that ALT is a good marker of this infection among pre-dialysis patients, in contrast to individuals under hemodialysis[17]. In one study, 39 pre-dialysis patients with chronic HCV infection were compared to HCV-infected hemodialysis subjects[43]. Pre-dialysis patients were older, showed a higher proportion of elevated aminotransferases levels, higher inflammatory activity and more advanced fibrosis on liver histology. However, since comparable fibrosis progression rates were observed, there is no evidence suggesting that chronic hepatitis C exhibits a more aggressive course in CKD subjects under conservative management. Interestingly, high HCV viral loads seems to be common in these patients[43], in contrast to what is

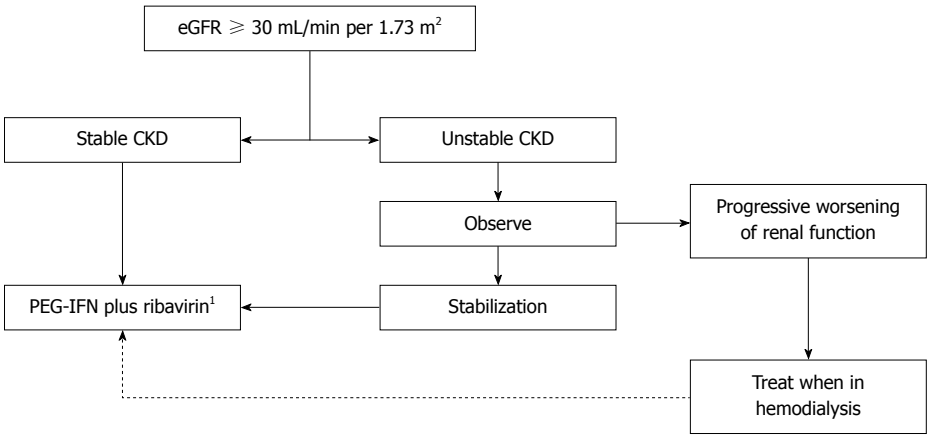

Figure 1 Treatment decision algorithm for hepatitis C virus-infected chronic kidney disease patients under conservative management. [1]Adjusted doses according to eGFR. eGFR: Estimated glomerular filtration rate; CKD: Chronic kidney disease; PEG-IFN: Pegylated interferon.

observed in hemodialysis subjects, who typically present low levels of serum HCV RNA, a finding possibly related to the clearance of HCV particles during dialysis[45-47].

Treatment decision relies on the CKD stage (based on eGFR), rate of progression of renal dysfunction and the possibility of a preemptive transplant. A treatment decision algorithm is proposed in Figure 1. Although antiviral therapy is feasible for subjects in all CKD stages, for most patients with CKD stage 4 (eGFR of 15 to 29 mL/min per 1.73 m^2) it is preferable to wait until there is indication for initiation of dialysis. This waiting attitude for CKD stage 4 patients is proposed only for those without significant liver fibrosis, considering the particularly low fibrosis progression rate and the poor tolerability of these subjects, as well as the high risk of further deterioration of kidney function and early indication for renal replacement therapy[48,49].

Treatment schedule consists of PEG-IFN α2a and ribavirin, with doses tailored to eGFR (Table 2), for 24 to 48 wk. Dose adjustment according to renal function is particularly needed for ribavirin[50], which concentrates in circulating red blood cells (RBCs)[51,52], causes a relative adenosine triphosphate deficiency and increased susceptibility to oxidative damage, leading to accelerated RBC turnover and hemolytic anemia[53]. Therefore, in CKD subjects, renal function and hemoglobin levels should be carefully monitored during antiviral therapy, due to the increased risk of ribavirin-induced anemia, which can be severe in patients who frequently have multifactorial anemia and other comorbidities (like coronary artery disease). The use of EPO (up to 40000 IU/wk) improves tolerability and promotes the stability of hematological parameters during treatment.

CKD patients under hemodyalisis

HCV infection is an important cause of morbidity and mortality in dialysis patients and has been associated with both liver disease-related deaths (due to complications of cirrhosis and hepatocellular carcinoma) and cardiovascular mortality[54-56]. It is possible that HCV contributes to atherogenesis through aggravation of metabolic syndrome factors and/or by leading to a chronic inflammatory state[57].

In CKD patients undergoing hemodialysis, HCV infection has distinct clinical and laboratory features as compared to the non-uremic population and KT recipients, which can affect the management of those subjects. The prevalence of advanced liver fibrosis is lower (4% to 10%)[58,59], and progression to cirrhosis during hemodialysis seems to be uncommon[60]. In addition, as mentioned above, for yet unknown reasons, ALT levels are lower than those observed in non-uremic patients, even in the presence of significant histological damage, which hampers its utility as a marker of HCV infection[18,61].

Anti-HCV has proven to be a reliable screening test for HCV chronic infection in CKD patients[62]. Although false-negative tests have been observed with first and second generation kits, this became rather unusual with third generation enzyme immunoassays and chemiluminescence assays[62,63].

It should be noted that, although these patients are immunocompromised due to the underlying disease, low HCV viral loads are typically observed[45-47]. The mechanisms involved in this phenomenon are poorly understood and are probably multifactorial. Filtration of viral particles into the dialysate, adherence of the virus to the surface of the dialysis membrane, and destruction of viral particles during the dialysis procedure have been proposed as potential mechanisms[46,47]. It is not clear whether the type of dialysis would significantly affect the clearance of HCV particles. However, it has been suggested that HCV viral load is lower in CKD patients under chronic hemofiltration[64]. Moreover, the phenomenon of intermittent HCV viremia, characterized by low levels of serum HCV viral load intercalated with episodes of undetectable HCV RNA, has been commonly reported in CKD patients under hemodialysis[46,65-68]. This event is responsible for false-negative results in HCV RNA assays in 33% to 67% of anti-HCV-reactive patients[46,65-68], which not only can result in delayed treatment (or no treatment at all), but

Table 3 Meta-analyses on the treatment of chronic hepatitis C in hemodialysis patients

Ref.	Year	Trials (*n*)	Therapy	*n*	SVR[1]
Russo *et al*[95]	2003	11	IFN	213	33% (21%-51%)
Fabrizi *et al*[96]	2003	14	IFN	269	37% (28%-48%)
Gordon *et al*[97]	2008	20	IFN	459	41% (33%-49%)
		5	PEG-IFN	87	37% (9%-77%)
Fabrizi *et al*[99]	2008	24	IFN	529	39% (32%-46%)
		4	PEG-IFN	116	31% (7%-55%)
Gordon *et al*[98]	2009	20	IFN	428	45%
Alavian *et al*[100]	2010	21	IFN	491	39% (32%-46%)
		12	PEG-IFN	279	39% (27%-52%)
Fabrizi *et al*[101]	2010	16	PEG-IFN	254	33% (24%-43%)
Fabrizi *et al*[102]	2011	10	PEG-IFN + RBV	151	56% (28%-84%)
Fabrizi *et al*[103]	2014	11	PEG-IFN + RBV	287	60% (28%-97%)

[1]Mean overall estimate for sustained virological response (range). IFN: Interferon; PEG-IFN: Pegylated interferon; RBV: Ribavirin.

also contributes to environmental transmission of HCV in dialysis units. Several physiopathogenetic mechanisms have been proposed to explain the intermittent HCV viremia, like heparin interference with the PCR assay used for the detection of HCV RNA[69], mechanical extraction of viral particles adhering to dialyzer membrane[47,70], and induction of interferon production, hepatocyte growth factor, or other cytokines with antiviral properties by the hemodialysis procedure[71-73].

Therefore, isolated undetectable results of HCV RNA should not be interpreted as absence of replication. To better clarify HCV viral kinetics in this population, it is recommended for all anti-HCV-positive CKD patients on hemodialysis to perform sequential HCV RNA monitoring by using a highly sensitive detection method like reverse transcriptase-polymerase chain reaction (RT-PCR) or transcription-mediated amplification[74-77].

Occult HCV infection could conceivably also represent a risk for nosocomial transmission of HCV within hemodialysis units, as well as an additional risk of reactivation and progressive liver disease after KT. However, a study evaluating 417 hemodialysis subjects found only a single case of HCV RNA detectable in peripheral blood mononuclear cells in the absence of HCV RNA in serum, suggesting that occult HCV infection is very rare in CKD patients in hemodialysis[63].

Although widely performed and accepted as the gold-standard method to evaluate hepatic fibrosis, liver biopsy is an invasive technique with associated morbidity[78]. CKD individuals frequently exhibit major hemostatic disorders and hemorrhagic complications, posing additional risks for patients undergoing invasive procedures[79]. Transjugular liver biopsy is an alternative procedure for obtaining liver specimens that has already been evaluated in the CKD population[80,81]. Although safe, this procedure is not widely available and frequently provides small samples, which might underestimate fibrosis staging. Hence, there is a need for the development of accurate noninvasive tests to estimate liver fibrosis, especially among dialysis patients, in whom a higher risk for liver biopsy complications has been observed in most, but not all, studies[80,82-84]. Noninvasive tests such as APRI (AST-to-platelet ratio index), FibroTest® and transient hepatic elastography have shown good diagnostic performance to predict the severity of liver fibrosis in hemodialysis patients with chronic hepatitis C, and can be used as alternative methods to liver biopsy for subjects with contraindications to the procedure or for those who refuse to be biopsied[58,85-87].

Hepatitis C eradication before KT theoretically improves survival and reduces the occurrence of chronic graft nephropathy[88], *de novo* glomerulonephritis[89] and post-transplant diabetes mellitus[90]. After transplantation, viremia increases significantly[9] and progression of liver fibrosis occurs[91,92], with an evident negative impact on survival after 10 years of transplantation[93]. Moreover, given the risk of treatment-induced graft dysfunction and poor tolerance of interferon-based therapy, antiviral therapy has limited indications in HCV-infected KT subjects[94]. Thus, in KT candidates, treatment should be offered regardless of the degree of histological injury, with the goal of viral eradication. It is highly recommended that common clinical comorbidities such as anemia, retinopathy and cardiovascular disease should be identified and controlled before treatment.

There have been several trials of hepatitis C treatment in hemodialysis patients, mostly uncontrolled and with different therapeutic regimens. These trials have been included in many meta-analysis[95-103], which are listed in Table 3.

Overall SVR rates derived from meta-analysis appear not to be very different for the use of standard IFN or PEG-IFN. However, in a randomized, controlled trial, viral load and use of PEG-IFN (*vs* standard IFN) were predictive of SVR[104]. The addition of ribavirin seems to provide a significant increase in SVR, but demands greater care in pretreatment evaluation and in monitoring and managing of anemia (including EPO supplementation). Studies evaluating combined therapy with interferon and ribavirin used ribavirin doses from 200 mg 3 times a week up to 300 mg/d[105-115]. Dropout rates were highly heterogeneous, ranging from 0% to 71%.

Given its easier dosing schedule and possible higher efficacy, it is recommended to use PEG-IFN (preferably PEG-IFN α2a 135 μg) once a week, after dialysis session, in combination with ribavirin. The ribavirin dose should be titrated according to patient tolerability, as follows: an initial dose of 200 mg once a week is given, followed by increments of 200 mg every two weeks until the maximum dose tolerated (stable levels of hemoglobin above 10 g/dL are often required). After stabilization of ribavirin dosage (usually between 400 to 1200 mg/wk), PEG-IFN is initiated and used for 24 to 48 wk (Figure 2).

HCV viral kinetics can be used to support clinical decisions during treatment. Early virological response has a positive predictive value of 67% to predict SVR

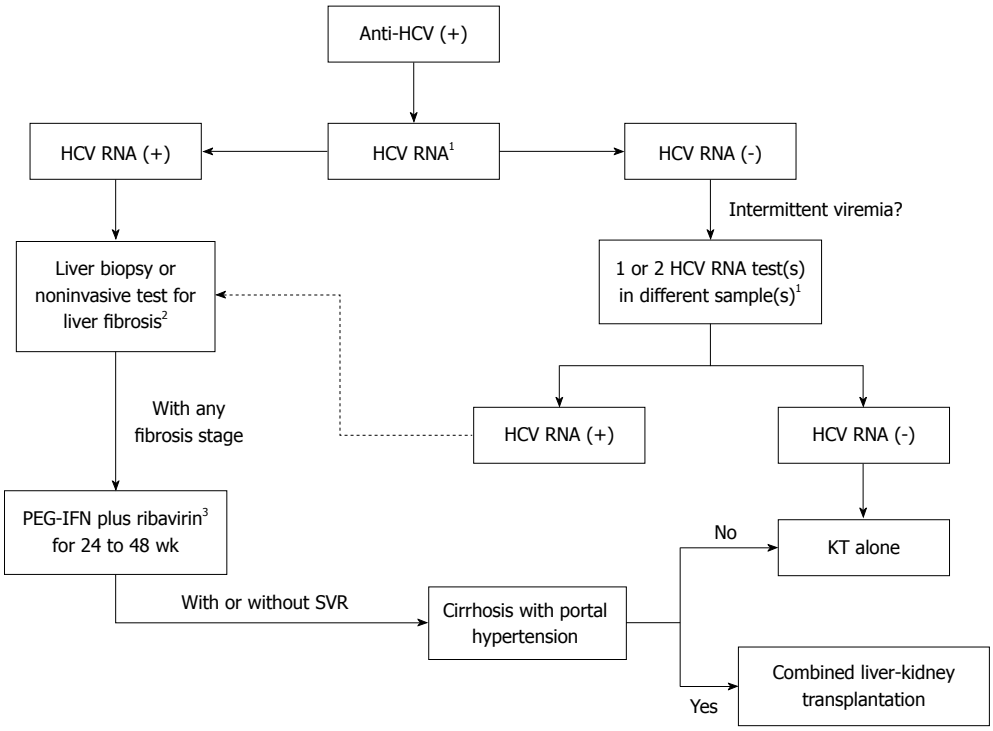

Figure 2 Treatment decision algorithm for hepatitis C virus-infected chronic kidney disease patients under hemodialysis who are candidates for kidney transplantation. [1]By real-time PCR or transcription-mediated amplification; [2]APRI, FibroTest or transient hepatic elastography; [3]Adjusted doses (see text for details). PEG-IFN: Pegylated interferon; KT: Kidney transplantation; SVR: Sustained virological response; HCV: Hepatitis C virus; KT: Kidney transplantation.

and a negative predictive value (NPV) of 75% in patients receiving interferon monotherapy[98]. More recently, it has been observed a NPV of 100% for SVR if HCV RNA is detectable on week 12 of treatment[116].

Preliminary reports have suggested that first wave HCV NS3/4A protease inhibitors telaprevir and boceprevir could be used in CKD patients, with good efficacy and safety profile[117-120]. There is no need for dose adjustments for telaprevir or boceprevir since dialysis does not exert a substantial influence on the pharmacokinetics of the drugs[121,122]. It is possible that the next generations of anti-HCV direct-acting antiviral agents (DAAs), such as second and third waves NS3/4A protease inhibitors, NS5A polymerase inhibitors, NS5B polymerase inhibitors and cyclophillin inhibitors will overcome the therapeutic barrier in this population, especially when interferon-free and ribavirin-free regimens become available.

Patients with cirrhosis, particularly those with portal hypertension, may have a decreased survival and increased morbidity after renal transplantation[123]. In these cases, a renal transplantation alone is contraindicated and combined liver-kidney transplantation should be considered. For patients with compensated cirrhosis and without significant portal hypertension, isolated renal transplant appears to be safe[124,125]. In these subjects with advanced fibrosis or cirrhosis, imaging monitoring and upper endoscopy are recommended for the screening of hepatocellular carcinoma and esophageal varices, respectively.

CHRONIC HEPATITIS C AFTER KIDNEY TRANSPLANTATION

Although some studies have failed to find a negative impact on clinical outcomes after KT[126,127], the majority of studies so far reported indicate that HCV infection is associated with increased liver-related mortality and fibrosis progression among HCV-infected KT patients, with a significant reduction in patient and graft survival, possibly related to accelerated fibrogenesis and increased liver damage induced by the use of immunosuppressive regimens[88,91-93,123,128-133]. Recent evidence also suggests that KT recipients with chronic HCV infection have an increased risk of post-transplant *de novo* glomerulonephritis[89,90,134], diabetes mellitus[90,135], and azathioprine hepatotoxicity[136].

In contrast to what is observed in CKD patients under dialysis, HCV-positive KT recipients more often present with false-negative anti-HCV results, even with newer immunoassays[63,137]. In a recent study, 19 out of 417 KT recipients were HCV RNA-positive and 3 of those patients (16%) were anti-HCV-negative by using chemiluminescence immunoassays[63]. This inability to mount an antibody response against HCV is probably related to the immunosuppressive therapy. Another consequence of immunosuppression is the significant increase in HCV viral load, with no reports of intermittent viremia so far[9,138]. Interestingly, similarly to hemodialysis subjects, there is a very low prevalence of occult HCV infection in KT recipients[63].

Table 4 Studies on the treatment of chronic hepatitis C in kidney transplant recipients

Ref.	Year	*n*	Therapy	Interferon dose	Duration (mo)	SVR
Harihara *et al*[154]	1994	3	IFN	3-6 MU *biw*	NA	NA
Therret *et al*[155]	1994	13	IFN	3-5 MU *tiw*	About 4	NA
Magnone *et al*[156]	1995	11	IFN	1.5-5.0 MU *tiw*	6	NA
Rostaing *et al*[157]	1995	14	IFN	3 MU *tiw*	About 5	0%
Ozgür *et al*[158]	1995	5	IFN	4.5 MU *tiw*	6	NA
Yasumura *et al*[159]	1997	6	IFN	6 MU *tiw*	About 7	33%
Durlik *et al*[160]	1998	11	IFN	3 MU *tiw*	About 6	0%
Hanafusa *et al*[161]	1998	10	IFN	9 MU *tiw*	6	10%
Tokumoto *et al*[162]	1998	6	IFN	9 MU *tiw*	6	50%
Baid *et al*[163]	2003	12	IFN + RBV	3 MU *tiw*	Variable	33%
Tang *et al*[164]	2003	4	IFN + RBV	3 MU *tiw*	12	50%
Shu *et al*[165]	2004	11	IFN + RBV	1 MU *tiw*	12	27%
Izopet *et al*[166]	1997	15	IFN	3 MU *tiw*	About 5	0%
Sharma *et al*[167]	2006	6	IFN + RBV	3 MU *tiw*	About 12	33%
Pageaux *et al*[168]	2009	8	PEG-IFNα2a	180 μg *qw*	6-12	50%
Aljumah *et al*[169]	2012	19	PEG-IFN + RBV	90-180 μg *qw*	12	42%
Sanai *et al*[170]	2013	32	PEG-IFN + RBV	135-180 μg *qw*	12	38%

IFN: Interferon; MU: Million units; *biw*: Two times a week; NA: Not available; *tiw*: Three times a week; PEG-IFN: Pegylated interferon; RBV: Ribavirin; SVR: Sustained virological response rate by intention-to-treat analysis.

There is still no consensus on the best immunosuppressive strategy in HCV-positive KT recipients. Antiviral activity of cyclosporine A (CsA), probably acting by antagonizing the effect of cyclophilin B on HCV replication[139], has been demonstrated both *in vitro* and *in vivo*[139-143], and it is possible that CsA may exert a beneficial effect on necroinflammatory activity in HCV-related liver disease among KT recipients[144-146].

Additional differences from hemodialysis patients are the higher prevalence of advanced liver disease in KT recipients[59,91,92], and the better accuracy of aminotransferases for the prediction of significant histological lesions[147-149]. Although liver biopsy is still recom-mended to evaluate the severity of hepatic lesions in patients on hemodialysis patients as well as in transplant recipients[150,151], several noninvasive methods for the assessment of liver fibrosis have been studied in HCV-positive KT patients, including simple blood tests[58], Fibro Test[85,152], and liver elastography[152,153], with fair diagnostic performances. In selected cases, these methods can be used as alternatives to liver biopsy. Screening for hepatocellular carcinoma and esophageal varices is indicated for patients with advanced fibrosis or cirrhosis.

As for antiviral therapy, there are several heterogeneous studies including small series of HCV-infected KT patients (ranging from 3 to 32 subjects), treated with standard or pegylated IFN alone or in combination with low dose ribavirin[154-170] (Table 4). Seventeen studies have been compiled in 2 meta-analyses[94,171], and the mean overall estimates for SVR with IFN monotherapy (10 studies), IFN plus ribavirin (4 studies) and PEG-IFN plus ribavirin (3 studies) were 16%, 36% and 43%, respectively.

Despite initial concerns about increased risk of graft dysfunction and loss, ranging from 0% to 40% in early studies[159-162,166], more recent series have shown lower graft rejection rates, between 0 and 5%[167-170]. Advances in immunosuppression therapy and the use of the less immunogenic PEG-IFN are possible explanations for this observation. However, dropout rates remain high (mean incidence of 28%) and do not differ greatly between early and recent studies[154-170], probably due to the worse anemia found in those receiving IFN in combination with ribavirin.

AASLD and KDIGO guidelines recommend against antiviral therapy for HCV-infected KT recipients, with the exceptions of fibrosing cholestatic hepatitis or life-threatening vasculitis[150,172]. Nevertheless, treatment with PEG-IFN and ribavirin should be considered for patients with advanced fibrosis, always taking into account time after transplantation, eGFR and renal function stability.

With the aid of EPO supplementation (doses up to 40000 IU/wk to maintain hemoglobin levels $\geqslant$ 10 g/dL), an initial ribavirin dose of 200 mg once a day is given, followed by increments of 200 mg every two weeks until the maximum dose tolerated, with target dosage of ribavirin defined according to eGFR (Table 2). After stabilization of ribavirin dosage (usually between 400 to 800 mg/d), interferon is initiated and used for 48 wk, irrespective of HCV genotype. Even in absence of comparative trials, it is suggested to use PEG-IFN (preferably PEG-IFN α2a 135-180 μg) once a week, in combination with ribavirin (see Figure 3). There are no studies supporting the use of DAAs for the treatment of KT HCV-positive recipients, but it is expected that these patients will benefit from interferon-free regimens.

SIDE EFFECTS OF ANTIVIRAL THERAPY

Treatment with IFN (standard or pegylated) and ribavirin is associated with frequent and sometimes serious side effects[173,174]. Among the latter are autoimmune diseases (worsening or *de novo* thyroid disorders, diabetes mellitus, psoriasis, *etc*.), significant hemolytic anemia and severe

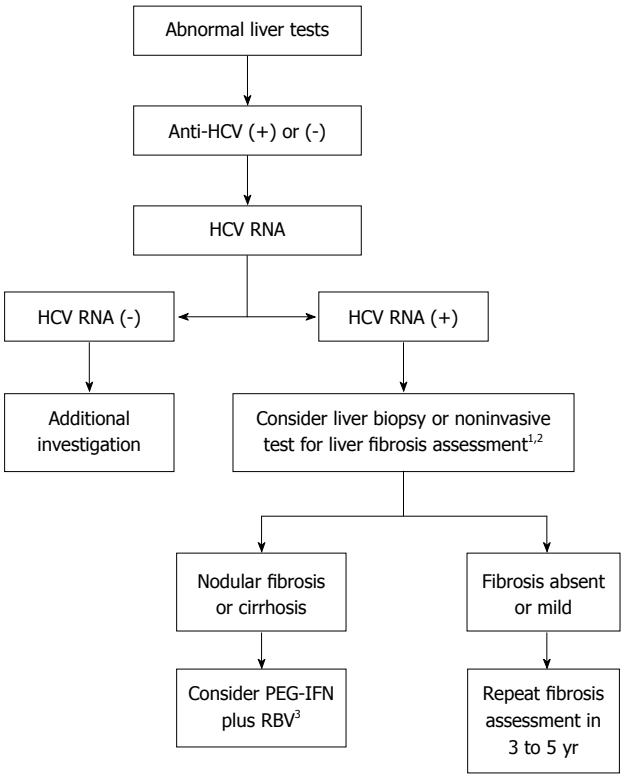

Figure 3 Treatment decision algorithm for hepatitis C virus-infected kidney transplantation recipients. [1]Particularly if time after transplantation > 5 years; [2]APRI, TX3, FibroTest or transient hepatic elastography; [3]Adjusted doses according to eGFR. PEG-IFN: Pegylated interferon; RBV: Ribavirin; eGFR: Estimated glomerular filtration rate.

depression. In a recent meta-analysis of eleven clinical studies published by Fabrizi *et al*[103], the summary estimate for dropout rate was 0.18 (95%CI: 0.08-0.35), with a large heterogeneity across studies, mainly due to anemia (24%) and infections (13%).

Except from hemolytic anemia, side effects are mainly related to IFN. The majority of the patients receiving IFN presents with a flu-like syndrome, characterized by diffuse myalgia, headache, fatigue and fever. Generally, these symptoms are self-limited and managed by common analgesics. Depression can be induced by IFN in 20% to 30% of the cases, usually after three months of treatment[175]. Being mild to moderate in intensity, IFN-induced depression can generally be handled with conservative measures, by non-psychiatrist professionals. However, if severe depression develops, HCV treatment must be stopped and the patient should be immediately referred to a psychiatrist. IFN-induced cytopenias (thrombocytopenia and leucopenia), are relatively common, typically dose-dependent and rarely associated with clinically significant complications, even in CKD patients. IFN dose reductions and the use of growth factors usually allow the continuation of therapy[173,174].

On the other hand, as previously discussed, the ribavirin-induced hemolytic anemia is very common and troublesome in CKD patients, due to its severity and potential noxious consequences in these subjects with high cardiovascular risk. Initial low doses of ribavirin, early dose reductions, and EPO supplementation are the main strategies for the management of anemia in this context. It should be mentioned that ribavirin can carry an increased risk of birth defects, and proper contraception during and up to six months after therapy must be adopted. Minor side effects like cough and skin rash also seem to be mostly associated with ribavirin[173,174].

With the first-generation HCV protease inhibitors (PIs), boceprevir and telaprevir, complex drug-to-drug interactions and tolerability issues remain a concern[176,177]. Boceprevir is associated with an increased incidence of anemia and dysgeusia and telaprevir is associated with an increased incidence of dermatological disorders, anemia, and anorectal symptoms[178-181]. An approximately 15% to 26% increase in anemia incidence in patients under triple therapy with boceprevir or telaprevir has been observed[178-181]. In these patients, anemia is considered the consequence of the combined effects of ribavirin-induced hemolysis and the bone marrow suppression of IFN and PI. In the same manner of dual therapy, ribavirin dose reductions and EPO are used for the management of anemia, although blood transfusions are also frequently required. Dysgeusia and anorectal symptoms are infrequently severe and often improve under conservative measures and dietetic modifications. A wide spectrum of dermatological disorders has been described in approximately 50% of the patients treated with first-generation PIs, particularly with telaprevir, ranging from simple pruritus with or without rash to severe skin reactions like Stevens-Johnson's syndrome or DRESS syndrome[182]. Emollients/moisturizers and topical corticosteroids are sufficient for most cases (90% to 95%), but dermatological consultations are frequently needed for more severe cases. Treatment discontinuation is required in about 6% of patients[182].

NEW PERSPECTIVES FOR HCV THERAPY IN CKD PATIENTS

In spite of the increment in SVR rates with the use of the first-generation PIs telaprevir and boceprevir in subjects with preserved renal function, the higher incidence of significant side effects (mainly severe anemia and dermatological reactions) and the frequent drug-drug interactions have hampered their widespread use in difficult-to-treat populations, such as CKD patients. Newer DAAs, like sofosbuvir (a nucleotide NS5B polymerase inhibitor), simeprevir (a second generation PI), and daclatasvir (a NS5A replication complex inhibitor), with or without PEG-IFN and/or ribavirin, or used in different combinations with one another, produces SVR rates superior to 90%[183-188]. Besides leading to the highest SVR rates ever seen, these emerging DDAs seems to exhibit a reduced potential for drug-drug interactions and a better safety profile, which will probably facilitate their use for the treatment of HCV infection in CKD subjects. However, the

appropriate dose of sofosbuvir has not been identified for subjects with severe renal impairment (eGFR < 30 mL/min per 1.73 m^2) or hemodialysis patients and dose adjustments might be necessary[189]. Likewise, although simeprevir is primarily metabolized by the liver and its renal elimination is minimal, the safety of the drug has not been evaluated in patients with CKD stages 4 and 5. Conversely, unpublished data suggested that dose reduction would not be needed for the use of daclatasvir in patients with any stage of renal impairment. Finally, a phase 3 study will evaluate the safety and efficacy of the all-oral and IFN-free combination therapy with ombitasvir (a NS5A replication complex inhibitor), ABT-450 (a second-generation PI) and dasabuvir (a non-nucleoside NS5B polymerase inhibitor), with or without ribavirin, for the treatment of genotype 1-infected CKD patients (ClinicalTrials.gov identifier NCT02207088)[190].

CONCLUSION

HCV infection is highly prevalent among CKD subjects and, consequently, in KT recipients, exerting a significant negative impact on clinical outcomes both before and after KT. Although interferon-based antiviral therapy in CKD patients is associated with poor tolerability and suboptimal efficacy, there has been mounting evidence that many patients can benefit from treatment. In those individuals, accurate characterization of liver disease and adequate assessment of comorbidities are mandatory for optimal management and therapeutic decisions.

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MINIREVIEWS

Magnetic resonance imaging based rectal cancer classification: Landmarks and technical standardization

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Abstract

Rectal cancer classification is important to determine the preoperative chemoradiation therapy and to select appropriate surgical technique. We reviewed the Western and Japanese rectal cancer classification and we propose our new classification based of Magnetic resonance imaging (MRI). We determine the relation of the tumor to fixed parameters in MRI, which are peritoneal reflection and levator ani muscle. Then, we classify the rectal cancer into four levels based on tumor distal margin and invasion to MRI parameters. We applied all three classifications to 60 retrospectively collected patients of different rectal cancer distance and we compared our classifications to the others. Based on each level we standardize our surgical approach. For stages Ⅰ-Ⅲ, We found that level Ⅰ where tumor distal margin is located above the peritoneal reflection and all of them were received low anterior resection (LAR) without chemoradiation. Level Ⅱ where tumor distal margin is located from the peritoneal reflection and above the levator ani insertion on the rectum. 90% of them were received LAR ± chemoradiation. Level Ⅲ where tumor distal margin is located at the level of levator ani insertion or invading any part of the levator ani. 60% of them had ULAR + coloanal anastomosis ± chemoradiation. Level Ⅳ where the tumor distal margin is located below the levator ani insertion; 77% were received APR ± chemoradiation. The overall kappa for all levels between surgeons and radiologist was 0.93 (95%CI: 0.87-0.99), which is indicating almost perfect agreement. We concluded that the management of rectal tumors differed among each tumor level and our new MRI based classification might facilitate the prediction of surgical and chemoradiation management with better communication among a multidisciplinary team comparing to other classifications.

Key words: Rectal cancer; Rectal classification; Surgical approach

Core tip: We reviewed the current rectal cancer classification and we propose a new rectal cancer classification based on new radiological parameters that might lead to change in the future decision making and management. We provide a comparison between our new novel classification, Western and Japanese one.

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INTRODUCTION

At the latter half of the 20th century, surgical therapy for rectal cancer underwent vital changes[1]. However, the complication and death rates from rectal cancer still remain high. This finding might be explained by variable application of the available therapies and surgeon decision at time of surgery. The variation in therapy use has been shown in rectal cancer compared with many other diseases[2-5]. Heald *et al*[6,7] and MacFarlane *et al*[8] started a "total mesorectal excision" technique as a method to reduce local recurrence rates (4%-8%) following rectal resection for rectal cancer, without adjuvant therapy. Surgeons with different types of training and institutions with variety of cancer patient's volumes provide rectal cancer management. Similar patients with similar tumors might receive different treatments depending on where and from whom they seek treatment; some of these treatment variations may represent suboptimal patient care[1].

An approach to management of rectal cancer patients using a multidisciplinary team (MDT) might provide better communication and facilitate high-quality management. It is proved in literature that the MDT could improve patient's 3- and 5-year survival[9,10]. The treatment strategy was altered after discussed at MDT meeting in 58.33% of colorectal cancer patients before operation especially in the matter of the sphincter-preservation and local control ($P = 0.049$)[10]. The issue of variability in surgical decisions among surgeons, particularly in low rectal cancer, to preserve the sphincter or perform advanced surgery remains unresolved world-wide. Several limitations present in the previous studies that have tried to found the nature of therapy variations. Large population-based studies (*i.e.*, the National Cancer Data Base reports from the American College of Surgeons Committee on Cancer and the American Cancer Society) show the variations in rectal cancer therapy over time without clinical interpretation of these variations[1].

The decision about surgery or chemoradiation treatment depends on several factors, one of them are radiologic evaluation of the tumor. Among those radiologic investigations, magnetic resonance imaging (MRI) commonly used to determine the status of perirectal node or the circumferential tumor margins[11-13]. However, small number of studies reported the relation between rectal cancer and peritoneal reflection or levator ani muscle by MRI[11,12,14,15]. Determination of the best preoperative surgical approach are depend on several factors one of them are tumor location in which, some authors depend on the height from anal verge while the others on radiologic relation to peritoneal reflection[14,15].

We reviewed the current tumor location classification and we propose a one based on the relationship of the tumor to fixed parameters on MRI imaging. Furthermore, we suggest the possible surgical approach based on the new classification.

ANATOMY

The management of rectal cancer poses many challenges to both surgeons and oncologists.

Knowledge of rectal anatomy is a key for medical and surgical management and important for the selection of appropriate imaging modality. The rectum begins immediately following the sigmoid colon, and ends at the anal canal. Based on distance from the anal verge; the rectum is divided into the upper (11-15 cm), the middle (7-10 cm), and the lower thirds (0-6 cm). The upper 1/3 is covered by peritoneum. The peritoneum covers only anteriorly at the middle rectum while the lower 1/2 is completely extraperitoneal[16,17]. The mesorectum behind the rectum separated from the presacral fascia by mesorectal fascia which its lack at the distal third of the rectum just before its entry in the pelvic floor muscles. In regards to the upper rectum, Benzoni *et al*[15] found that the relation between tumor location and peritoneal reflection is a prognostic factor in rectal cancer. The tumor located at the extra-peritoneal part of the rectum is more aggressive than those at the intra-peritoneal even when treated by neoadjuvant chemoradiotherapy[15,18,19].

The lower part of the rectum where the mesorectum end and levator ani muscle insert is an important part mainly for treatment decision, which is different from the part with mesorectum and above the levator ani insertion site. The tumor at this level can easily goes outside the rectal wall to the levator ani muscle or to the sphincters below this level, which might lead to change in chemoradiation and surgical approach.

RADIOLOGY

To optimize the treatment strategy on an individual basis, we need detailed information about primary tumor location, local extension, potential nodal-stage, potential circumferential resection margin involvement and extra-mural venous invasion[20]. The complexity of the anatomy and relationship of the tumor to adjacent structures, *i.e.*, bone and muscles-might lead to difficulty in prediction and management decisions regarding the type of surgical approach and chemoradiation use. Radiology plays a key role in tumor management. It provides a vital knowledge about the tumor diagnosis and preoperative staging. Of all the radiologic modalities that evaluate the rectal cancer, MRI is a superior modality that provides a better anatomical visualization comparing to computed tomography (CT) and endorectal ultrasound (EUS)[21]. In addition, it provides high accuracy in detection

of tumor location, tissue characterization, detailed anatomical relation to the tumor and tumor staging. So, preoperative MRI is useful modality to determine the surgical approach and need for neoadjuvant or adjuvant therapy[21].

The benefit of MRI for surgical treatment decisions was investigated retrospectively by Shihab *et al*[22] who found that MRI could objectively confirm the clinical impression by delineation of the local extent of the tumor and its relationship to the levator ani and the intersphincteric plane.

The accuracy of predicting tumor extent beyond the muscularis propria was within 0.5 mm tolerance in the mid or upper rectum, and suggests MRI can accurately predict ultimate outcome. MRI can also accurately measure the distance between the anorectal junction and/or and the distal part of the tumor and the luminal length of the tumor, circumferential resection margin particularly in the mid-rectum, involvement of the levator in the low rectum and the extramural depth of invasion[20,23].

CURRENT RECTAL CLASSIFICATIONS

Surgical approach and chemoradiation therapy decisions in treatment of rectal cancer were determined by multiple factors. Tumor location and preoperative stage are the most important clinical elements. In population-based studies, the information of the tumor location is rarely available. However, in the institution-specific studies, which usually provide more clinical data, cannot reflect the practices in a general population. A consensus statement for tumor location and how it affects surgical decisions differ between Japan and Western countries.

Currently, the tumor distance from the anal verge (upper, middle, and lower)[24,25] as adopted by Western and most others countries, or the relationship of the tumor to the peritoneal reflection (Ra, Rb, and P)[26] as proposed by Japanese surgeons, are used to determine tumor locations. Peritoneal reflection separates the Ra and Rb border, which approximately corresponds to the level of the middle Houston valve.

In regard to western classification, some studies reported that the tumor height from the anal verge might have beneficial on the radiotherapy of rectal tumors[1]. However, measurements of distances from the anal verge are still unclear due to the methods provides to date like digital rectal examination or rigid sigmoidoscopy, are rather vague and subjective and the reported distances from the anal verge to the levator ani insertion and peritoneal reflection are variable[27]. Accordingly, based on this landmark we cannot measure the exact location of the peritoneal reflection or level of levator ani muscle insertion. We considered that if the peritoneal reflection and levator ani insertion could be clearly visualized and localized radiologically, that would provide a more objective localization method rather than the distances from the anal verge measurement.

In regard to Japanese classification, it is based on the relation with respect to peritoneal reflection. However, still it is difficult to determine preoperatively the exact location of the peritoneal reflection. Furthermore, in relation to the mesorectum, the definitions of extra-peritoneal and intra-peritoneal locations are vague[27]. The start of the P level, which is the anal canal, is not clearly defined preoperatively by specific fixed landmarks.

WHY WE NEED NEW CLASSIFICATION?

Preoperative evaluations are vital to determine the treatment options for rectal cancer. Moreover, the decision about the preoperative chemoradiotherapy and type of surgery is dependent on tumor location, tumor invasion, nodal status, involvement of the meso-rectal fascia, and distant metastasis[11-13,20]. Due to the changes of the surgical approach of the rectal cancer over several years, new rectal classification to predict the best approach are needed. In the era of sphincter preservation, technical innovations and improvement in the radiological modalities, the surgical approach for each tumor location is not clearly defined.

SURGICAL APPROACH

Previously rectal cancer was treated either by anterior resection (AR) or abdominoperineal resection (APR). Then low anterior resection (LAR) proves non-inferiority results to APR with better quality of life. Currently, the low rectal cancer can be approached by ultra low anterior resection with coloanal anastomosis, partial or complete intersphincteric resection, tailored levator ani excision (hemilevator excision) or even local excision. Those approaches proved to be alternative to APR with better sphincter saving and quality of life. However, preoperative MRI might predict which of those approaches are more likely to be used but lacking of specific parameters encourage us to propose a new rectal classification.

CHEMORADIATION APPROACH

High-resolution pelvic MRI is now routinely used in Korea as well as in United Kingdom and Europe as a preoperative staging and selection tool for the use of preoperative chemoradiation. MRI can easily localize the tumor above or below the peritoneal reflection and strongly predicts the likelihood of involvement of the circumferential resection margin, involvement of the levator ani muscle in the low rectum and the extramural depth of invasion. Furthermore, it can identify patients at risk of the surgeon being unable to achieve an R0 resection[23].

Long course chemoradiation or short course radiation therapy are routinely used for locally advanced rectal cancer "T3, T4 tumors or any TN+ (stage Ⅱ, Ⅲ)" as defined by National Comprehensive Cancer Network

guidelines, however; recent improvements in the quality of surgery, *i.e.*, TME, MRI and pathological reporting of the operative specimen, lead most of the investigators to question both these approaches[20]. Most of the time long course chemoradiation used for low rectal cancer in a goal to preserve the sphincter but definition of low rectal cancer are variable and some patients considered as low rectal cancer based on tumor distance from anal verge while the exact location of the tumor are above the levator ani where using short course radiation or TME alone may be better to avoid radiation complications. The same thing can be applied to peritoneal reflection, which is an important since most of the tumor above the peritoneal reflection less likely to have local recurrence where the radiation can be omitted. But the exact location of peritoneal reflection needs to be determined by MRI and not by only measuring the distance from anal verge where from this point we propose our new classification.

OUR PROPOSED CLASSIFICATION

Our proposed classification depends on division of the rectum into levels which is depends on a fixed parameters seen on preoperative MRI. Those parameters are peritoneal reflection and levator ani insertion on the rectum.

In a study by Jung *et al*[27] found that based on the location of the peritoneal reflection, the subdivision of the rectum by MRI is more objective and anatomical than other classification methods and could facilitate treatment planning. However, his classification do not cover the whole rectum for that we add a levator ani insertion as a parameter for lower rectal classification.

Methods

MRI (sagittal and coronal views) was used to determine fixed, tumor-related anatomical parameters and so initiate a change in the management plan. These parameters were peritoneal reflection and levator ani muscle insertion.

To locate the tumor in the rectum, we determined two factors - the tumor "distal margin" from the parameters and tumor tethering "radiologically the tumor closely in contact with adjacent structure and we cannot clearly define a separate margin" or "invasion" to those parameters.

We then defined each rectal division "class" and performed a retrospective study of a 60 rectal cancer patients selected randomly based on their tumor distance from anal verge and comparison between our classification and those used by Western countries and Japan were performed too. We showed the advantage of our classification in the prediction of the exact surgical procedure over the others.

Data were analyzed using the SPSS statistical software (Statistical Product and Service Solutions version 18 for Windows; SPSS Inc., Chicago, IL, United States). A P value less than or equal to 0.05 was deemed to indicate statistical significance. The tumor level on MRI compared to sigmoidoscopy by the 1 radiologist and 2 colorectal surgeons. The inter-observer agreement between surgeon and surgeon and surgeons and radiologist was evaluated using Cohen's Kappa statistics. Kappa statistic was tested for overall levels and each level separately. Weighted Kappa < 0 indicate (no agreement); Kappa = 0.0 - 0.20 (slight agreement); Kappa = 0.21 - 0.40 (fair agreement); Kappa = 0.41 - 0.60 (moderate agreement); Kappa = 0.61 - 0.80 (substantial agreement); Kappa = 0.81 - 1.00 (almost perfect agreement). Statistical analyses were performed using Stata-MP 10.1 (Stata Corp, College Station, Tex).

Results

Based on the MRI sagittal view, the first parameter was the rectal peritoneal reflection, which is the line connecting the lowest point of the peritoneal reflection anteriorly to the highest point of the sacral promontory posteriorly (Figure 1).

Based on the MRI coronal view, the second parameter was the levator ani insertion on the rectum (anorectal ring) (Figure 1).

Thus, based on those parameters, we divided the rectum into the following four levels: (1) Level Ⅰ: the tumor "distal margin" is located "above" the peritoneal reflection on the sagittal MRI view (Figure 1A); (2) Level Ⅱ: the tumor "distal margin" is located "from" the peritoneal reflection and "above" the levator ani insertion on the rectum (anorectal ring) on MRI sagittal and coronal views (Note: the tumor should not invade the levator ani at this level) (Figure 1); (3) Level Ⅲ: the tumor "distal margin" is located "at" the level of levator ani insertion on the rectum (anorectal ring) or the tumor margin is invading any part of the levator ani from its origin to its insertion (Figure 2); and (4) Level Ⅳ: the tumor "distal margin" is located "below" the levator ani insertion on the rectum (Figures 3 and 4).

To apply our classification clinically, we performed a retrospective comparative analysis of 60 randomly selected patients diagnosed with rectal cancer at various locations within the rectum. Thirty-eight (63%) of these patients were male, and 22 (36%) were female. Their mean age was 59.18 ± 12.66 years. Twenty-seven patients (45%) received neoadjuvant chemoradiotherapy. The preoperative tumor stage was determined. A total of six patients (10%) had a disease of stage Ⅰ, 10 (16%) had stage Ⅱ, and 44 (73%) had stage Ⅲ. The postoperative stage was also reported. A total of 6 (10%) patients had stage 0 (complete response) disease, 9 (15%) had stage Ⅰ, 15 (25%) had stage Ⅱ, and 30 (50%) had stage Ⅲ.

Then, we compared the tumor location on MRI and sigmoidoscopy for each patient. Of 60 patients, 12 showed a difference of 2 cm or more. However, this finding was not statistically significant (P = 0.64). Therefore, we depended on the MRI view, which could show not only the tumor distance from the anal verge but also the anatomical landmarks and relationships of the tumor to the parameters.

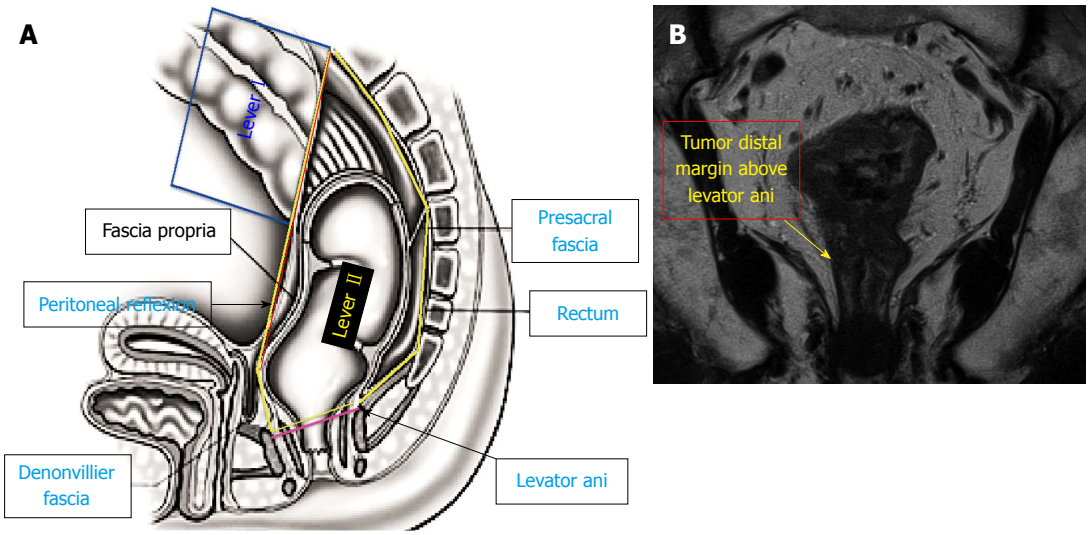

Figure 1 Levels Ⅰ and Ⅱ (A), level Ⅱ (tumor distal margin above levator ani muscle insertion) (B).

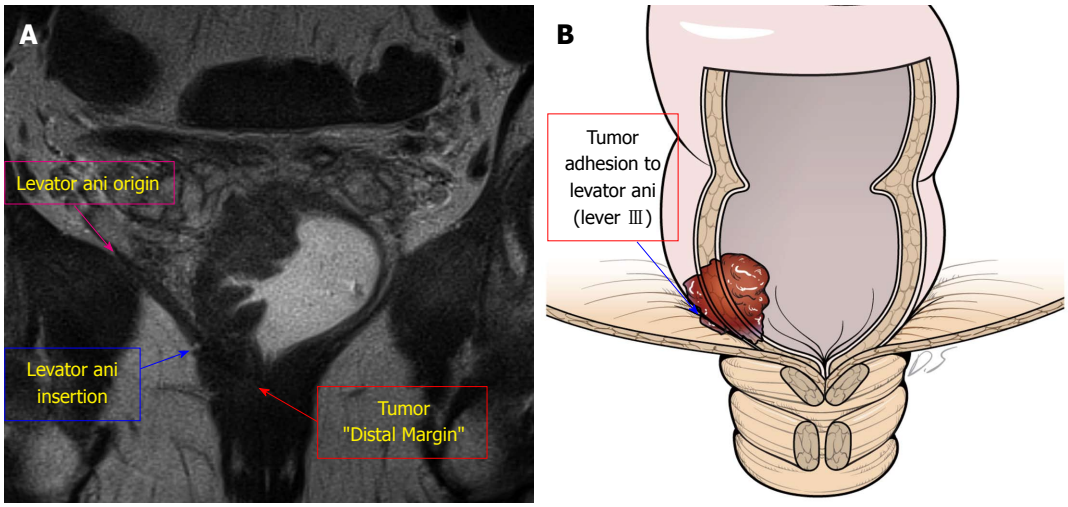

Figure 2 Level Ⅲ (tumor distal margin at the level of levator ani insertion) (A), Level Ⅲ (B).

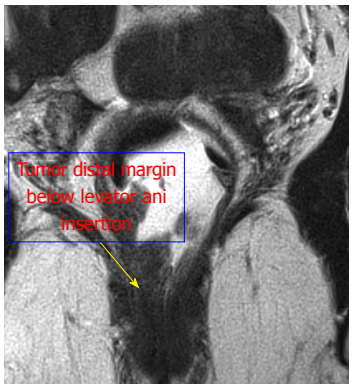

Figure 3 Level Ⅳ (tumor distal margin below levator ani insertion).

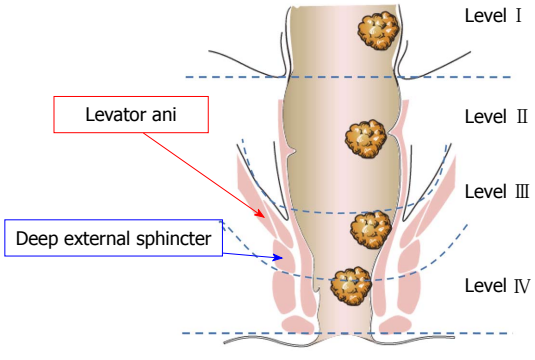

Figure 4 All tumor levels.

We next compared our tumor location level to the Western and Japanese rectal location divisions. For the Western division, we selected random cases to cover all parts of the ano-rectum (upper, middle, lower) from 1 to 15 cm. For the Japanese division, we divided the rectum based on the location above the peritoneal reflection (Ra), below the peritoneal reflection (Rb), and at the anatomical anal canal (P).

Table 1 Upper rectal cancer data

Age	Sex	CCRT	CM	R	L	Pre OP TS	TNM	O/L/R	OP	Post OP TS	TNM
67	M	Y	11	Rb	2	Ⅲ	T3N+	L	LAR	Ⅲ	YPT3N1
68	F	Y	11	Rb	2	Ⅲ	T3N+	O	LAR	Ⅲ	YPT3N1
56	F	N	11	Rb	2	Ⅲ	T3N+	L	LAR	Ⅲ	PT3N1
70	F	N	11	Rb	2	Ⅲ	T2N+	L	LAR	Ⅲ	PT3N1
67	F	Y	12	Rb	2	Ⅲ	T3N+	L	LAR	Ⅱ	YPT3N0
48	M	Y	12	Rb	2	Ⅲ	T3N+	L	LAR	Ⅱ	YPT3N0
49	F	N	12	Rb	2	Ⅲ	T4N+	O	LAR	Ⅲ	PT4N1
50	F	N	12	Rb	2	Ⅲ	T3N+	L	LAR	Ⅰ	PT1N0
52	M	Y	13	Ra	2	Ⅱ	T3N0	L	LAR	Ⅱ	YPT3N0
60	M	N	13	Ra	2	Ⅲ	T3N+	O	LAR	Ⅱ	PT3N0
64	M	N	13	Rb	2	Ⅲ	T3N+	R	LAR	Ⅲ	PT3N1
74	M	N	13	Rb	2	Ⅲ	T3N+	L	LAR	Ⅲ	PT3N1
54	F	N	14	Ra	2	Ⅲ	T3N+	O	LAR	Ⅲ	PT3N1
67	M	N	14	Ra	2	Ⅲ	T3N+	L	LAR	Ⅲ	PT4N1
63	M	N	14	Ra	1	Ⅲ	T3N+	L	LAR	Ⅱ	PT3N0
44	F	Y	15	Rb	2	Ⅲ	T4N+	O	Hartm.	Ⅲ	YPT4N1
66	M	N	15	Ra	1	Ⅲ	T4N+	O	LAR	Ⅲ	PT3N1
60	M	N	15	Ra	1	Ⅲ	T3N+	L	LAR	Ⅲ	PT3N2
73	M	N	15	Ra	1	Ⅱ	T3N0	R	LAR	Ⅱ	PT3N0

CCRT: Concurrent chemoradition therapy; R: Rectal Japanese class; L: Level class; OP: Operative; TS: Tumor stage; TNM: Tumor, lymph node, metastasis; O/L/R: Open/laparoscopic, robotic), only M0 tumors were included; LAR: lower anterior resection.

For upper rectal cancer (Table 1), the tumor distal margin of 19 patients (31%) was 11-15 cm. Eight of them were Ra, and 11 were Rb. Four of them were level Ⅰ, and 15 were level Ⅱ. Clinically, 17 (89%) had stage Ⅲ disease, and two (10%) had stage Ⅱ disease. Six patients had neoadjuvant chemoradiation. Six patients received open surgery, two received robotic surgery, and eleven received laparoscopic surgery. Eighteen (94%) patients had lower anterior resection (LAR), whereas one (5%) patient underwent the Hartmann procedure due to tumor invasion of other organs; this was level Ⅱ. Postoperatively, one patient (5%) was stage Ⅰ, 6 (31%) were stage Ⅱ, and 12 (63.1%) were stage Ⅲ. Technically, we found that all level I patients received LAR.

For middle rectal cancer (Table 2), the tumor distal margin in 16 patients (26%) was 7-10 cm. All of these patients were Rb located in level Ⅱ. Clinically, 1 (6%) patient was stage Ⅰ, 4 (25%) were stage Ⅱ, and 11 (68%) were stage Ⅲ. Half of the patients (50%) received neoadjuvant chemoradiation therapy. Four patients underwent surgery using an open approach, and six underwent each laparoscopic and robotic surgery. All patients (100%) had LAR. Postoperatively, one patient was each of stage 0 and Ⅰ. Six patients were stage Ⅱ, and eight were stage Ⅲ.

For lower rectal cancer (Table 3), the tumor distal margin in 26 (43%) patients was 1-6 cm. All patients were P according to the Japanese classification. According to our classification, 11 (42%) patients were at level Ⅱ, five (19%) were at level Ⅲ, and 10 (38%) were at level Ⅳ. Clinically, five (19%) patients were stage Ⅰ, four (15%) were stage Ⅱ, 16 (61%) were stage Ⅲ, and one (3%) was stage Ⅳ. Half (50%) of the patients received neoadjuvant chemoradiation. Nine patients underwent robotic and open surgery each, whereas eight underwent laparoscopic surgery. Because three levels exist in those considered to have low-rectal tumors, the procedures also differed. Nine (34%) patients had LAR and APR each. Six (23%) patients had ultra-low anterior resection and hand-sewn coloanal anastomosis (CAA). Two (7%) patients had ultra-low anterior section with intersphincteric resection (ISR) and hand-sewn CAA. Complete pathologic response (stage 0) was achieved in 5 (20%) patients. Seven (26%) showed a stage Ⅰ disease, 3 (11%) had stage Ⅱ, and 10 (40%) had stage Ⅲ.

Overall procedures for level Ⅰ 4 (100%) patients had LAR, for level Ⅱ 38 (90%) patients had LAR, 3 (7%) ULAR + CAA and 1 (2%) had Hartman procedure. For level Ⅲ 3 (60%) patients had ULAR + CAA and 1 (20%) had LAR and APR each. For level Ⅳ 7 (77%) patients had APR and 2 (22%) had ULAR + ISR.

The overall kappa for all levels between surgeons and radiologist was 0.93 and confidence interval (CI: 0.87-0.99), which is indicating almost perfect agreement. The kappa for level Ⅰ was 1 (100%), which is, indicate a perfect agreement between surgeons and radiologist. Regarding level Ⅱ the kappa between surgeons was 1 (100%) but between surgeons and radiologist was 97.61% with overall average kappa of 0.98 (98.41%) and still indicates a perfect agreement. For level Ⅲ the kappa between surgeons was 1 (100%) but between surgeons and radiologist was 80% with overall average kappa of 0.86 (86.66%), which is indicate a perfect agreement. For level Ⅳ the kappa was 1(100%) between all observers and indicates almost perfect agreement.

SURGICAL DECISION BASED ON THE NEW CLASSIFICATION

Our new rectal parameters and classification could provide a common understanding among individuals in

Table 2 Middle rectal cancer data

Age (yr)	Sex	CCRT	CM	R	L	Pre OP TS	TNM	O/L/R	OP	Post OP TS	TNM
64	F	N	7.0	Rb	2	Ⅱ	T3N0	O	LAR	Ⅱ	PT3N0
68	F	N	7.0	Rb	2	Ⅲ	T3N+	O	LAR	Ⅱ	PT3N0
50	M	Y	7.2	Rb	2	Ⅲ	T3N+	L	LAR	Ⅲ	YPT3N2
85	M	Y	7.8	Rb	2	Ⅲ	T3N+	R	LAR	Ⅲ	YPT3N1
66	M	N	8.0	Rb	2	Ⅲ	T3N+	O	LAR	Ⅲ	PT3N1
73	M	N	8.0	Rb	2	Ⅲ	T3N+	L	LAR	Ⅲ	PT3N1
49	M	Y	8.5	Rb	2	Ⅲ	T3N+	R	LAR	0	YPT0N0
58	M	Y	8.8	Rb	2	Ⅲ	T3N0	L	LAR	Ⅱ	YPT3N0
78	M	N	9.0	Rb	2	Ⅱ	T3N0	L	LAR	Ⅲ	PT4N1
52	F	N	9.3	Rb	2	Ⅲ	T3N+	R	LAR	Ⅲ	PT3N2
61	F	Y	9.5	Rb	2	Ⅱ	T3N0	L	LAR	Ⅲ	YPT3N1
73	F	Y	9.5	Rb	2	Ⅲ	T3N+	R	LAR	Ⅱ	YPT3N0
48	M	Y	10.0	Rb	2	Ⅰ	T2N0	R	LAR	Ⅰ	YPT2N0
74	F	Y	10.0	Rb	2	Ⅲ	T2N+	R	LAR	Ⅱ	YPT3N0
47	F	N	10.0	Rb	2	Ⅲ	T3N+	O	LAR	Ⅲ	PT3N2
82	M	N	10.0	Rb	2	Ⅱ	T3N0	L	LAR	Ⅱ	PT3N0

CCRT: Concurrent chemoradition therapy; R: Rectal Japanese class; L: Level class; OP: Operative; TS: Tumor stage; TNM: Tumor, lymph node, metastasis; O/L/R: Open/laparoscopic, robotic), only M0 tumors were included; LAR: lower anterior resection.

Table 3 Lower rectal cancer data

Age (yr)	Sex	CCRT	CM	R	L	Pre OP TS	TNM	O/L/R	OP	Post OP TS	TNM
51	M	Y	1.0	P	4	Ⅲ	T4N+	O	APR	Ⅲ	YPT2N1
57	M	Y	1.0	P	4	Ⅲ	T3N+	R	ULAR + I SR	0	YPT0N0
49	M	N	1.0	P	4	Ⅲ	T3N+	O	APR	Ⅲ	PT4N2
75	M	N	1.3	P	4	Ⅱ	T3N0	O	APR	Ⅰ	PT2N0
37	M	Y	2.0	P	4	Ⅲ	T4N+	O	APR	Ⅲ	YPT3N1
62	F	Y	2.0	P	4	Ⅲ	T3N+	O	ULAR + I SR	0	YPT0N0
54	M	N	2.0	P	4	Ⅱ	T3N0	O	APR	Ⅱ	PT3N0
48	M	N	2.0	P	4	Ⅱ	T3N0	O	APR	Ⅲ	PT3N1
83	F	Y	3.0	P	3	Ⅳ	T4N+	O	APR	Ⅱ	YPT3N0
75	F	Y	3.5	P	2	Ⅲ	T3N+	R	LAR	0	YPT0N0
54	M	N	3.5	P	3	Ⅰ	T2N0	R	ULAR + CAA	Ⅰ	PT1N0
72	M	N	3.7	P	4	Ⅲ	T3N+	L	APR	Ⅲ	PT3N2
37	M	Y	4.0	P	3	Ⅲ	T3N+	L	ULAR + CAA	0	YPT0N0
47	F	Y	4.0	P	2	Ⅲ	T3N+	L	LAR	0	YPT0N0
60	M	N	4.2	P	2	Ⅰ	T1N0	L	LAR	Ⅰ	PT1N0
27	F	N	4.3	P	2	Ⅱ	T3N0	R	LAR	Ⅰ	PT2N0
64	F	Y	5.0	P	2	Ⅲ	T2N+	R	LAR	Ⅱ	YPT3N0
65	M	N	5.0	P	2	Ⅰ	T2N0	L	ULAR + CAA	Ⅰ	PT2N0
37	M	Y	5.5	P	3	Ⅲ	T3N+	L	ULAR + CAA	Ⅲ	YPT3N2
71	F	N	5.6	P	2	Ⅲ	T2N+	R	LAR	Ⅰ	PT1N0
50	M	Y	5.8	P	3	Ⅲ	T3N+	R	LAR	Ⅲ	YPT2N2
40	M	N	6.0	P	2	Ⅰ	T2N0	L	ULAR + CAA	Ⅰ	PT2N0
56	M	N	6.0	P	2	Ⅰ	T2N0	L	LAR	Ⅲ	PT3N2
44	M	Y	6.1	P	2	Ⅲ	T3N+	R	ULAR + CAA	Ⅲ	YPT3N1
56	M	Y	6.3	P	2	Ⅲ	T3N+	R	LAR	Ⅲ	YPT3N1

CCRT: Concurrent chemoradition therapy; R: Rectal Japanese class; L: Level class; OP: Operative; TS: Tumor stage; TNM: Tumor, lymph node, metastasis; O/L/R: Open/laparoscopic, robotic), only M0 tumors were included; LAR: lower anterior resection; CAA: Coloanal anastomosis.

a multidisciplinary team. Whenever the tumor level is identified, the most likely procedure and chemoradiation choice can be determine directly.

Regarding upper rectal cancer, the term "upper rectal cancer" does not indicate the location of the tumor above or below the peritoneal reflection, so decisions regarding chemoradiation therapy cannot be made based only on this term. Additionally, even with 11-15 cm, the peritoneal reflection is located at a variable distance from the anal verge from patient to patient, and many radiation oncologists do not recommend administration of radiation to tumors above the peritoneal reflection.

The Ra values (indicating tumors above the peritoneal reflection and that are level Ⅰ) for both the Japanese and our classifications were similar, indicating a tumor above the peritoneal reflection that is less likely to be treated with radiation therapy unless a T4 lesion is evident. However, Ra defined as a tumor above peritoneal reflection. Some surgeons include those tumors with distal margin invading the peritoneal reflection, which

might denial the use of chemoradition. Those tumor invading the peritoneal reflection considered high risk for recurrence, therefore; in our classification we include them in the level Ⅱ.

Concerning middle rectal cancer, most advanced cases at this level would receive chemoradiation therapy. However, determining the exact distance of tumors located at 7-11 cm had no much impact on the surgical decision.

Regarding the Japanese and our classifications, level Ⅱ was most similar to Rb, but we limited our level to tumors with distal margins above the levator ani insertion. We found that all patients at level Ⅱ had LAR with or without chemoradiation.

Thus, all lesions at level Ⅱ can be treated using LAR with or without chemoradiation. However, some lesions with invasion to other organs (T4) and not responding to chemoradition therapy should be treated using pelvic exenteration, if possible.

Regarding lower rectal cancer, most advanced cases at this level require chemoradiation for sphincter preservation and to reduce local recurrence. However, determining the exact distance of tumors located at 1-6 cm did not reflect the surgical procedure performed unless the surgeons were unfamiliar with sphincter preservation, in which case the patients underwent APR. Moreover, the distance of the levator ani insertion to the rectum differed among patients. Some patients with a tumor distance of 5 or 6 cm had LAR, whereas APR or CAA with or without ISR was performed in others. Those differences were due to the variability in the location of the levator ani. If the tumor is located at 5 or 6 cm above the levator ani, LAR is highly possible with stapler anastomosis. However, if the tumor is located at or below the levator ani, stapler anastomosis could not be performed, and perineal dissection was conducted instead.

In the Japanese classification, all tumors located at the anal canal are referred to as P. However, in our classification, we subdivided this area into two levels due to the variability of the technique: levels Ⅲ and Ⅳ. Level Ⅲ; in which tumors are located at the level of the levator ani or are invading it (level of the dentate line) require careful dissection to achieve safe circumferential margins. If there is no invasion to the levator ani, CAA with or without ISR is the treatment of choice; otherwise, partial (or tailored) levator excision or APR is used for invasive tumors. Additionally, this area is technically challenging due to the muscular structure and location of the anorectal ring.

For level Ⅳ, in which the distal margin of the tumor is below the levator ani (the tumor originates from the rectum but extends down to the anal canal), most early stage tumors can easily undergo CAA with or without ISR. However, in cases of sphincter invasion, APR is the procedure of choice. Level Ⅳ is not technically challenging and is easier than level Ⅲ due to the proximity to the anal verge; additionally, circumferential margins can be achieved easily.

Thus, in tumors with a distal margin located at or below the level of levator ani, stapling anastomosis is less likely to be possible, and perineal phase dissection must be conducted whether hand-sewn anastomosis is performed with or without ISR, or with APR.

In our study, of 25 patients with a tumor located at 1-6 cm, 17 (68%) had a tumor located at 3-6 cm. Although the tumors were located below 6 cm (low rectal cancer), 11 (64%) were level Ⅱ. Eight (72%) of these 11 tumors were treated using LAR, whereas the other three (27%) underwent hand-sewn CAA. Those three cases underwent colo-anal anastomosis directly because the surgeon did not attempt to locate the tumor intraoperatively after complete rectal mobilization.

After the localization of the levator ani and its relationship to the tumor has been determined, whether a procedure should be performed above (LAR with stabling anastomosis) or below (APR/CAA ± ISR) it can be determined.

Neoadjuvant chemoradiation therapy can change the tumor characteristics and might lead to tumor partial or complete response. Also it leads to increase the rate of sphincter saving procedure. In addition to that the tumor location can be changed. With all those factors we with Jung *et al*[27] and we need to go with tumor level to know which procedure will be suitable for each individual patient based on restaging MRI. Summary of the Management of Rectal Tumors: (1) for early stage lesions, either local excision is used or the guidelines of radical therapy are followed; (2) for radical resection of tumors at stages T1-3, N±: Level Ⅰ: LAR, - chemoradiation; (3) for radical resection of tumors at stages T1-3, N ±: Level Ⅱ: LAR/± chemoradiation; (4) for radical resection of tumors at stages T1-3, N±: Level Ⅲ: CAA ± ISR, partial levator ani or sphincter resection, APR/± chemoradiation; and (5) for radical resection of tumors at stages T1-3, N±: Level Ⅳ: APR or CAA ± ISR/± chemoradiation.

CONCLUSION

Management of rectal tumors differed among levels. However, our new radiological classification based on MRI facilitates determination of the most appropriate tumor management technique at each level of the rectum, and might increase communication among individuals in a multidisciplinary team. Evaluation of this classification in a prospective study is warranted.

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Gastric foregut cystic developmental malformation: Case series and literature review

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Abstract

Foregut cystic developmental malformation (FCDM) is a very rare lesion of the alimentary tract, especially in the stomach. We discuss the concepts of gastric duplication cyst, bronchogenic cysts, and FCDM. Nomenclature has been inconsistent and confusing, but, by some definitions, gastric duplication cysts involve gastric mucosa and submucosal glands, bronchogenic cysts involve respiratory mucosa with underlying cartilage and glands, and FCDM lacks gastric mucosa or underlying glands or cartilage but has pseudostratified ciliated columnar epithelium (PCCE). We searched our departmental case files from the past 15 years and identified 12 cases of FCDM in the alimentary tract. We summarize the features of these 12 cases including a report in detail on a 52-year-old man with a submucosal cyst lined with simple PCCE and irregular and stratified circular muscle layers that merged with gastric smooth muscle bundles near the lesser curvature of the gastric cardia. A literature review of cases with this histology yielded 25 cases. We propose the term gastric-FCDM for such cases. Our own series of 12 cases confirms that preoperative recognition of the entity is infrequent and problematic. The rarity of this developmental disorder, as well as a lack of understanding of its embryologic origins, may contribute to missing the diagnosis. Not appreciating the diagnosis preoperatively can lead to an inappropriate surgical approach. In contrast, presurgical recognition of the entity will contribute to a good outcome and reduced risk of complications.

Key words: Endoscopic ultrasound-guided fine-needle aspiration; Foregut duplication cyst; Gastric duplication cyst; Laparoscopic surgery; Pseudostratified columnar ciliated epithelium

Core tip: Gastric foregut cystic developmental malformation is a rare lesion that has been reported intermittently in recent decades. Its classification was inconsistent. It has often been misdiagnosed preoperatively. By missing the nature of the diagnosis, the surgical management was quite different. Through a review of the case series and literature concerning their clinical and radiologic features, and recognition of its embryologic and histological origin, we found that it is not an irregular disease and is an easily missed diagnosis. It can be cured by rational surgery, contributing to a good outcome and reduced risk of complications.

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INTRODUCTION

Gastric foregut cystic developmental malformation (G-FCDM) is a rare lesion and is composed of an intra-mural cyst in the stomach with a lining of pseudostratified ciliated columnar epithelium (PCCE). Cysts of this nature have been reported for several decades and were given various names including duplication cyst of the stomach with ciliated lining[1-4], bronchogenic cyst of the stomach[5-12], and foregut duplication cyst of the stomach[13-21]. Preoperatively, a misdiagnosis as gastrointestinal stromal tumor (GIST) and leiomyoma was not unusual[2,8,9,11,12,15]. With the aforementioned shared histopathologic characteristics and clinicoradiologic features that mimic GIST, are cysts with PCCE truly a form of gastric duplication cyst[4]? Recent reanalysis has led to the conclusion that a cystic developmental malformation of the primitive foregut vestiges may be a reasonable embryologic explanation for the entity[14,22,23]. In this paper, we review the features of the aforementioned gastric cysts and review those that only have PCCE, along with a series of secondary changes arising in the developmental process of the cyst, which could help with choosing the appropriate surgical procedure[24].

CLINICAL SUMMARY

One month prior to admission, a 52-year-old man had epigastric discomfort and noted a mass. As he did not have chills, fever, nausea, vomiting, or diarrhea, he did not attach importance to it initially. However, his symptoms persisted, prompting him to seek medical attention. Endoscopy of the upper gastrointestinal tract revealed a gastric submucosal eminence at the subphrenic gastroesophageal junction. Pathologic diagnosis was chronic nonatrophic gastritis. Abdominal frontal and transversal computed tomography (CT) showed a well-circumscribed, homogeneous, non-enhancing, low-density, submucosal cystic mass measuring 3.0 cm × 4.2 cm on the lesser curvature of the stomach near the cardia, with a CT number of 17 Hu (Figure 1). Preliminary suspicion was of a GIST with cystic change. On physical examination, he was in good condition and laboratory studies were within the normal range. He had an exploratory laparotomy under general anesthesia. Intraoperatively, the liver, peritoneum, and pelvis were free of metastatic disease, and no ascites was detected. A soft 4.0 cm × 3.0 cm mass was noted at the lesser curvature, near the cardia. He underwent proximal gastrectomy with lymph node dissection. The postoperative course was uneventful and there was no recurrence after 5 mo.

PATHOLOGIC FINDINGS

Gross examination of the proximal stomach showed that the cystic lesion was embedded in the gastric muscular layer and intimately associated with the submucosal lesser curvature near the cardia. It was located towards the esophageal margin of the proximal gastrectomy specimen (Figure 2). Thick, pale-yellow liquid was present within the cyst. The cyst did not communicate with the gastric lumen and measured 6.5 cm × 5 cm × 5 cm with a wall thickness that ranged from 0.1 to 0.3 cm.

The cyst wall was lined by a simple columnar epithelium and had a criss-crossing and stratified circular muscle layer (Figure 3A), and part of the cystic wall was lined with irregular longitudinal muscle bundles (Figure 3B). This circular muscle was stratified and merged with the muscular wall of the stomach at the attachment site, and the myenteric plexus was seen (Figure 4). Cartilaginous tissue, seromucinous glands, gastric epithelium and submucosal glands were not identified. Squamous metaplasia of the PCCE was detected (Figure 5). Cholesterol crystals and a histocytic response were present. All the dissected systematic lymph nodes were negative.

EMBRYOLOGY AND HISTOLOGY

G-FCDM may represent a congenital anomaly with late differentiation rather than imperfect involution of embryonic vestiges, but the undifferentiated foregut vestiges undergo transition and differentiate during the embryonic period[20]. One model postulates that the primitive lung bud derives from the respiratory laryngotracheal tube of the ventral foregut but is incompletely separated from the dorsal foregut in week seven of fetal development[24]. Several hypotheses to explain the dissociated foregut malformations suggest that they probably arise from pinching off and form the budding remnants[25], migration of the aberrant rest, supernumerary lung buds, and incomplete involution of the connecting stalk or fistula that connects with the

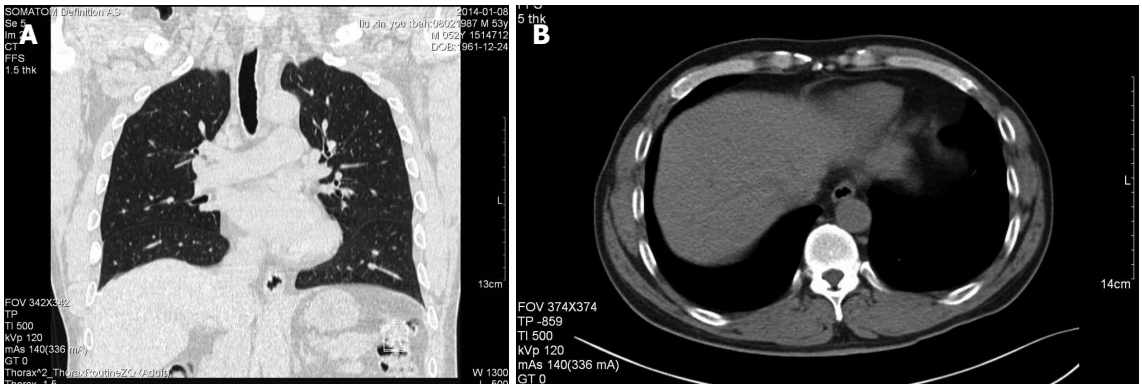

Figure 1 Computed tomography. A: Frontal abdominal contrast-enhanced computed tomography (CT); B: Transversal abdominal CT demonstrating a homogeneous, low-density and well-circumscribed, subserosal cystic mass on the lesser curvature of the gastric cardia.

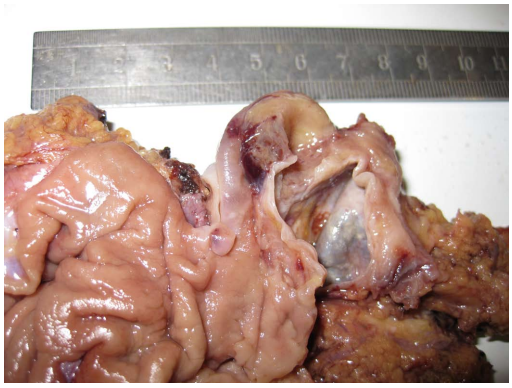

Figure 2 Gross appearance of the resected specimen of proximal gastrectomy. A cyst measured 6.5 cm × 5 cm was embedded in the gastric muscular layer, and did not communicate with the gastric lumen.

digestive or respiratory tract[26-28].

Some authors would classify our described case as gastric bronchogenic cyst. Bronchogenic cysts are thought to be in the spectrum of foregut cystic malformations. Traditional embryology theory postulates that the basic difference between the two entities is the timing of budding. Foregut cysts are derived from pinching off at the time of bronchiolar differentiation, which is later than that of bronchogenic cysts; hence, the presence of cartilage and glandular tissue in the wall of the bronchogenic cyst[29]. Histologically, the foregut cysts are lined with PCCE, subepithelial connective tissue followed by a smooth muscle layer and an outer fibrous layer[29,30], but bronchogenic cysts additionally contain cartilage and glandular tissues in the cyst wall[16,23].

Gastrointestinal duplication cysts are rare congenital malformations that may occur anywhere from the mouth to the anus[31-33]. Cunningham *et al*[19] have suggested that the term gastric duplication implies the presence of gastric epithelium. Ladd and Grossa[34], later supported by Parker *et al*[35], have proposed more detailed criteria: close proximity to the gastrointestinal tract; a lining that resembles some part of the gastrointestinal tract; and a smooth muscle layer that shares the muscle wall with the gut, or is intermingled with the muscular layer of

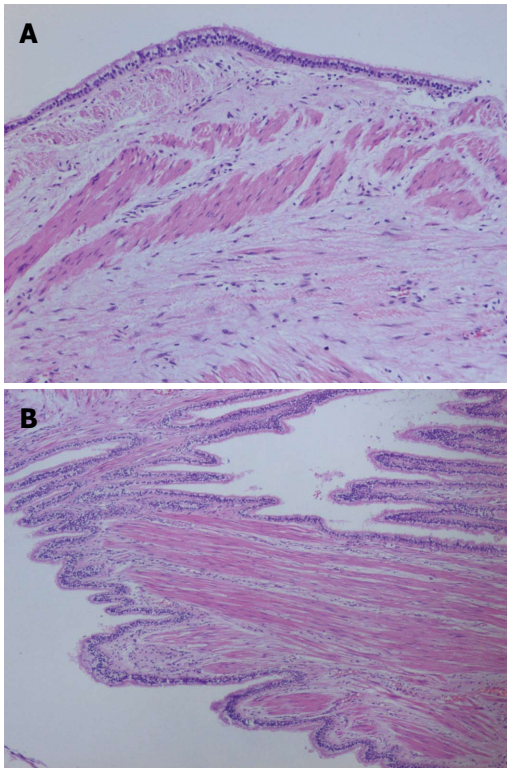

Figure 3 Submucosal cystic lesion. Hematoxylin and eosin staining showing the cyst wall lined by pseudostratified ciliated columnar epithelium (A) and submucosal cystic wall with irregular longitudinal muscle bundles (B), magnification × 200.

the bowel. Abiding by these criteria, cysts lined with PCCE do not qualify as gastrointestinal duplication cysts, including our 12 cases, because they lack archenteric epithelium. Similarly, in our cases, neither cartilaginous tissue nor seromucinous glands were present, so they do not qualify as bronchogenic cysts. In the literature, most reported cysts lined with PCCE are often described as foregut duplication cysts of the stomach[19].

CLINICAL FEATURES

Our literature search gathered 24 reports including 25

Table 1 Summary of gastric foregut cystic developmental malformation

No.	Sex	Age (yr)	Complaints	Location	Size (cm)	Ref.
1	M	56	No	NGEJ, AW	5 × 3 × 3	Napolitano *et al*[13], 2013
2	F	34	EP, GR	NGEJ, GC	4.5 × 3.2	Montemurro *et al*[1], 2011
3	M	29	AP	Fundus GC	8.5 × 5.5 × 4.8	Khoury *et al*[14], 2011
	F	26	EP	Middle body LC	5 × 2.2 × 2	
4	M	76	No	NGEJ, LC	4 × 4	Jiang *et al*[2], 2011
5	M	42	Left lumbar pain	AGIJ, LC	4.5 × 5.2	Mardi *et al*[15], 2010
6	F	25	EP	Gastric fundus	3 × 2.5 × 2	Jiang *et al*[5], 2010
7	F	60	No	Cardia, LC	3	Sato *et al*[6], 2008
8	F	72	No	Middle body, LC	2 × 1.5	Murakami *et al*[16], 2008
9	M	37	EP	NGEJ, LC	4 × 4	Wakabayashi *et al*[7], 2007
10	M	40	ED	NGEJ, LC	6 × 5	Hall *et al*[17], 2007
11	F	46	Vomiting	PW of fundus; Gastrosplenic ligament	8 × 5.5 3 × 3	Theodosopoulos *et al*[18], 2007
12	F	38	No	Cardia, LC	7 × 5	Lee *et al*[8], 2006
13	F	63	Fever, AP	PW of fundus	10 × 7.6	Cunningham *et al*[19], 2006
14	F	39	No	Fundus	4 × 2.5 × 1	Melo *et al*[9], 2005
15	M	26	EP	NA	NA	Rubio *et al*[10], 2005
16	F	62	No	NGEJ, LC	3.5 × 2.5 × 1.5	Song *et al*[11], 2005
17	F	59	No	PW of stomach, LC	7 × 5	Hedayati *et al*[12], 2003
18	M	35	EP	NGEJ, LC	7 × 6 × 5	Kim *et al*[20], 2000
19	M	34	No	GC	large	Ikehata *et al*[3], 2000
20	M	25	No	PW of fundus	6.5 × 5 × 5	Takahara *et al*[4], 1996
21	F	35	EP, nausea	PW	5.5 × 2.5 × 2	Laraja *et al*[21], 1995
22	F	61	Heart failure	Cardia, intramural	2 × 1.5	Shireman[36], 1987
23	F	46	No	NGEJ, GC	6 × 8	Gensler *et al*[22], 1966
24	M	52	ED	LC, NGEJ	6.5 × 5	Present case

AGIJ: Anterior of gastrointestinal junction; AW: Anterior wall; AP: Abdominal pain; ED: Epigastric discomfort; EP: Epigastric pain; GC: Greater curvature; GR: Gastroesophageal reflux; LC: Lesser curvature; NA: Not available; NGEJ: Near gastroesophageal junction; PW: Posterior wall.

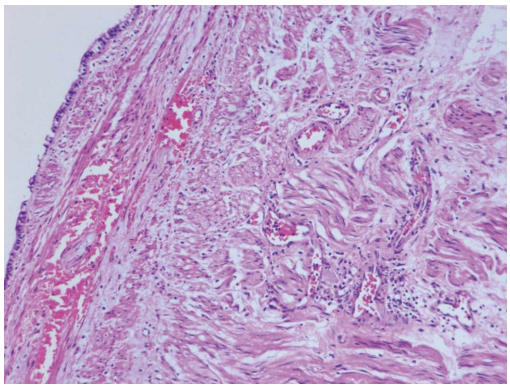

Figure 4 Regular, double-stratified, circular and longitudinal smooth muscles of the cyst and well-developed muscle layers continuous with gastric smooth muscle bundles, cartilaginous tissue, seromucous gland, or gastric epithelium were not identified. Hematoxylin and eosin staining (magnification × 200).

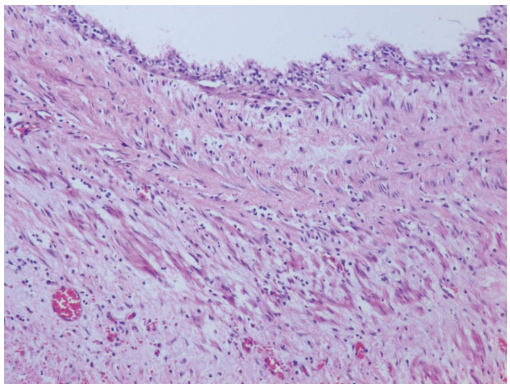

Figure 5 Squamous metaplasia tendency of the pseudostratified ciliated columnar epithelium. Hematoxylin and eosin staining (magnification × 200).

cases listed in Table 1[1-22,36]. From the literature review, gastric foregut cyst lined simply by PCCE is a delayed-onset disease (14 women and 11 men from 25 to 76 years-old). Our own series of patients in Table 2 also consisted of adults (age: 32-83 years) and had an equal sex distribution (6 women and 6 men). Most of the previously reported lesions involved the lesser curvature of the stomach and were near the gastroesophageal junction or cardia (12/24; 50%). Our series had a slight predominance of esophageal lesions (5/12; 42%), which was consistent with the literature reports of lesions above the diaphragm involving the esophagus[2,16]. Whether the predilection site of esophagus is related with the closest adjacency between the ventral embryo vestiges and dorsal tubes remains unknown. It is usually asymptomatic and occasionally found as a gastric wall mass on physical examination. Some patients present with epigastric pain. Patients with an older age or with a longer clinical history of symptoms tend to present with a larger mass and are more likely to have epigastric discomfort, gastric ulcer, gastroesophageal reflux, or occasionally canceration[37,38]. Some of these signs and symptoms are presumably related to the effect of the mass on adjacent structures[20]. Morphologically, the lined epithelium with focal squamous metaplasia were also sporadically reported[4,22].

Table 2 Foregut cystic developmental malformation of alimentary tract in our recent 15 years

No.	Sex	Age (yr)	Complaints	Location	Size (cm)	Surgical option
1	F	35	CT	Esophagus	2.5 × 1.5 × 1	CE
2	M	51	ED	Lower esophagus	3 × 2	CE
3	F	44	No	PT, lesser omental sac	8 × 3 × 2	CE
4	M	76	No	NGEJ, LC	4 × 4	Total gastrectomy with SLND
5	F	54	ED	Lower esophagus	4 × 2.5 × 2	CE
6	M	40	NA	Biliary tract	3 × 2.5	CE
7	F	42	CT	Esophagus	4 × 2.5 × 1	CE
8	F	32	No	PT, gastrosplenic ligament	5 × 4 × 3	CE
9	M	41	No	PW, LC	3.5 × 2.5 × 0.8	CE
10	F	42	NA	Esophagus	2.5 × 1.8	CE
11	M	83	NA	Distal ileum	6.7 × 5 × 4.1	CE
Present	M	52	ED	LC, NGEJ	6.5 × 5	Proximal partial gastrectomy with SLND

CE: Cyst excision; CT: Chest tightness; ED: Epigastric discomfort; LC: Lesser curvature; NA: Not available, NGEJ: Near gastroesophageal junction; PT: Pancreatic tail; PW: Posterior wall; SLND: Systematic lymph node dissection.

As a result of the cyst location within the gastric muscular layer and a lack of communication with the gastric lumen, many such lesions are preoperatively misdiagnosed as intramural GIST and leiomyoma, which present with different imaging findings, although they probably share similar clinical representation. It has been proposed that all foregut developmental anomalies, including gastric duplication cyst with PCCE lining and bronchogenic cyst without cartilage and glandular tissue, should be grouped under the heading of foregut cystic malformations because they all share a common origin from the foregut and differ from each other in migration, location, and degree of differentiation[23,27,28,39]. Gastric cysts with PCCE are not true duplication cysts of the foregut, but the cystic development of foregut embryologic vestiges. Therefore, we suggest designating this as G-FCDM. With regard to the predominant location of G-FCDM at the lesser curvature of the stomach, it remains to be established whether this is due to migration of the embryo vestiges or some other reason.

IMAGING FEATURES AND DIAGNOSIS

In symptomatic and occasionally discovered G-FCDM, CT can detect the presence of the abdominal mass, but it frequently fails to recognize its cystic nature due to the thick cyst wall[8]. Despite the fact that GIST is clinically more common than G-FCDM, it does not often show necrosis and cystic change. In the imaging study, cystic changes in GIST tend to be focal with irregular internal surfaces rather than smooth as in congenital cysts, and usually do not involve the whole tumor. Moreover, the proteinaceous cyst fluid[16,40] of G-FCDM is very helpful in identifying the necrosis of GIST. G-FCDM, but not GIST, can alter their shape with changing posture when they are large enough and with low tension. Leiomyoma is similar. Endoscopic ultrasound (EUS) is helpful in identifying the intramural or extramural relation of the gastrointestinal tract[41-45]. CT[46,47], magnetic resonance imaging or ultrasonography could indicates the presence of an abdominal cystic lesion or mass incidentally, but it cannot identify the nature of the lesion[2,21,40,48,49]. EUS-guided fine-needle aspiration (EUS-FNA)[50], CT-guided needle biopsy and intraoperative frozen section diagnosis can provide histologic diagnosis of G-FCDM and guide operative plans. The presence of PCCE and absence of neoplastic cells confirm the nature of the cyst[43], but considering the complications, some people do not advise performing a biopsy to confirm the diagnosis of resectable GIST because it can lead to tumor dissemination or hemorrhage[51,52].

THERAPIES

The management of asymptomatic cases remains controversial[41]. Watchful waiting is suggested after confirming the benign nature of these cysts by EUS-FNA, and Ponder and Collins[19] concluded that surgery is not necessary if the respiratory-type epithelial cells are diagnosed on EUS-FNA. For single symptomatic cases, the recommended management is complete cyst excision without violation of the gastric lumen[53]. Segmental or total gastrectomy is only a secondary alternative in the case of an indefinite diagnosis before operations[54]. However, if the cyst communicates with the gastric lumen that can easily induce infection, or with other serious gastric mucosal complications, such as ulceration, perforation, bleeding[55], fistula formation[38], obstruction and even malignant change, although rare, partial gastrectomy may be required[2,5,16]. From Table 2, we can see surgical treatment typically involved excision of the lesion without injury to attached organs, except the stomach. Of our three cases of G-FCDM, only one was correctly identified preoperatively and the cyst was successfully removed laparoscopically. The other two cases were incorrectly treated as GISTs, which are more common than congenital cysts, and led to unnecessary segmental gastrectomy and systematic lymph node dissection. With advances in medical technology and further understanding of G-FCDM, the advisable laparoscopic surgery for cyst removal has become more common in recent studies[16,24].

CONCLUSION

In summary, G-FCDM lined by PCCE is a rare lesion derived from foregut developmental malformation. The clinical manifestation is usually nonspecific, and it is easily misdiagnosed radiologically and clinically as a GIST or leiomyoma. EUS-FNA/CT-guided needle biopsy and frozen section diagnosis could be helpful in identifying the nature of the cyst and guide the surgical options. Although rare, better understanding of the origins of G-FCDM lined by PCCE and taking precise auxiliary examinations could help differential diagnosis from gastric wall masses, and surgically cure them without overtreatment.

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ORIGINAL ARTICLE

Basic Study

Increased density of tolerogenic dendritic cells in the small bowel mucosa of celiac patients

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Author contributions: Vorobjova T and Uibo R contributed equally to the research design; Vorobjova T performed the research, including immunohistochemical and immunofluorescence studies and microscopy, and analyzed the data and wrote the paper; Uibo O and Heilman K were responsible for the collection of clinical material, performed the gastroduodenoscopy and revised and approved the final version of the article; Uibo R guided and supervised the research and the drafting and editing of the final revision of the article.
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Abstract

AIM: To investigate the densities of dendritic cells (DCs) and FOXP3$^+$ regulatory T cells (Tregs) and their interrelations in the small bowel mucosa in untreated celiac disease (CD) patients with and without type 1 diabetes (T1D).

METHODS: Seventy-four patients (45 female, 29 male, mean age 11.1 ± 6.8 years) who underwent small bowel biopsy were studied. CD without T1D was diagnosed in 18 patients, and CD with T1D was diagnosed in 15 patients. Normal small bowel mucosa was found in two T1D patients. Thirty-nine patients (mean age 12.8 ± 4.9 years) with other diagnoses (functional dyspepsia, duodenal ulcer, erosive gastritis, *etc*.) formed the control group. All CD patients had partial or subtotal villous atrophy according to the Marsh classification: Marsh grade Ⅲa in 9, grade Ⅲb in 21 and grade Ⅲc in 3 cases. Thirty-nine patients without CD and 2 with T1D had normal small bowel mucosa (Marsh grade 0). The densities of CD11c$^+$, IDO$^+$, CD103$^+$, Langerin (CD207$^+$) DCs and FOXP3$^+$ Tregs were investigated by immunohistochemistry (on paraffin-embedded specimens) and immunofluorescence (on cryostat sections) methods using a combination of mono- and double-staining. Sixty-six serum samples were tested for IgA-tissue transglutaminase (tTG) using a fully automated EliA™ Celikey® IgA assay (Pharmacia Diagnostics, Freiburg, Germany).

RESULTS: The density of CD11c$^+$ DCs was significantly increased in CD patients compared with patients with normal mucosa (21.67 ± 2.49 *vs* 13.58 ± 1.51, P = 0.007). The numbers of FOXP3$^+$ cells were significantly higher in CD patients (10.66 ± 1.50 *vs* 1.92 ± 0.37, P = 0.0002) and in patients with CD and coexisting T1D (8.11 ± 1.64 *vs* 1.92 ± 0.37, P = 0.002) compared with patients with normal mucosa. The density of FOXP3$^+$ cells significantly correlated with the histological

grade of atrophic changes in the small bowel mucosa according to the March classification (r = 0.62; P < 0.0001) and with levels of IgA antibody (r = 0.55; P < 0.0001). The densities of IDO$^+$ DCs were significantly higher in CD patients (21.6 ± 2.67 *vs* 6.26 ± 0.84, P = 0.00003) and in patients with CD and coexisting T1D (19.08 ± 3.61 *vs* 6.26 ± 0.84, P = 0.004) compared with patients with normal mucosa. A significant correlation was identified between the densities of IDO$^+$ DCs and FOXP3$^+$ T cells (r = 0.76; P = 0.0001). The mean values of CD103$^+$ DCs were significantly higher in CD patients (10.66 ± 1.53 *vs* 6.34 ± 0.61, P = 0.01) and in patients with CD and associated T1D (11.13 ± 0.72 *vs* 6.34 ± 0.61, P = 0.00002) compared with subjects with normal small bowel mucosa. The mean value of Langerin$^+$ DCs was higher in CD patients compared with persons with normal mucosa (7.4 ± 0.92 *vs* 5.64 ± 0.46, P = 0.04).

CONCLUSION: The participation of diverse DC subsets in the pathological processes of CD and the possible involvement of tolerogenic DCs in Tregs development to maintain intestinal immunological tolerance in CD patients are revealed.

Key words: CD11c$^+$, CD103$^+$, IDO$^+$, Langerin (CD207$^+$) dendritic cells; FOXP3$^+$ regulatory T cells; Small bowel mucosa; Immunohistochemistry; Immunofluorescence; Celiac disease; Type 1 diabetes; Children

Core tip: Significantly higher densities of CD11c$^+$ dendritic cells (DCs) and of tolerogenic IDO$^+$, CD103$^+$ and Langerin$^+$ DCs in the small bowel mucosa of patients with celiac disease (CD) compared with subjects with normal small bowel mucosa were revealed using immunohistochemistry in 74 patients. This article highlights the participation of diverse DC subsets in the pathological processes in the small bowel mucosa, pointing out the importance of Langerin$^+$ DCs in untreated CD patients with and without type 1 diabetes and indicating the possible involvement of tolerogenic DCs in regulatory T cells development to maintain intestinal immunological tolerance in CD patients.

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INTRODUCTION

Celiac disease (CD) is characterized by an altered immune response to ingested wheat gluten and related prolamins in rye and barley, which leads to inflammation, villous atrophy and crypt hyperplasia in the small bowel mucosa, accompanied by an increased number of infiltrating lymphocytes in the epithelium and in the lamina propria (LP)[1]. The generation of inflammatory and damaging T cells is governed by several control mechanisms, while intestinal dendritic cells (DCs) are the key cells that regulate which T cells become activated or deleted. In this process, regulatory T cells (Tregs) also play an important role in intestinal homeostasis[2,3].

The role of the gut immune system is also thought to be of crucial importance in the pathogenesis of type 1 diabetes (T1D)[4-6]. Several research groups have demonstrated a marked association between the development of T1D and preceding alterations in the small bowel mucosa, including altered gut microbiota in these patients[4,7,8]. As a result, CD and T1D coexist more frequently compared with chance occurrence, with an average prevalence of CD among children with T1D of 4.5% (0.97%-16.4%) in 26 reports[9].

DCs have received much attention in CD due to their strategic role in gut homeostasis by processing external antigens (including wheat proteins) and by determining tolerance to self-antigens[10,11]. The important role of CD11c$^+$CD103$^+$ DCs in the induction of Tregs differentiation has been established[12]. CD103$^+$ DCs are required for the induction of tolerogenic immune responses and contribute to the control of inflammatory responses and homeostasis in the intestinal mucosa by the incremental conversion of naive T cells into FOXP3$^+$ Treg cells[13,14]. Functionally specialized CD103$^+$ DCs derived from the small intestinal LP appear to be the only cells able to regulate T cells homing[15].

In addition to the role of CD103$^+$ DCs, that of indoleamine 2,3-dioxygenase (IDO) for Tregs induction was also demonstrated[16-18]. IDO is an immunomodulatory enzyme involved in tryptophan catabolism with immunosuppressive effects that has been implicated in the control of intestinal inflammation[17]. Higher IDO expression has been measured in intestinal biopsies from CD patients[19].

Among the DCs covering body surfaces, either mucosa or skin, DCs carrying Langerin (CD207) proteins have received considerable interest[20]. Langerin was originally identified as a Langerhans cell (LC)-specific C-type lectin receptor involved in antigen capture[21]. Langerin expression is predominant in skin DCs, but Langerin-expressing DCs are also present in the mucosal tissue and can be induced by immunization and sometimes by nutrient deficiency[22]. The expression of Langerin by CD103$^+$CD11b$^+$ LP DCs in the human ileum has recently been reported[23]. However, the presence of Langerin$^+$ DCs in the small bowel mucosa in pathological conditions such as CD and T1D has not yet been studied. Still, one could preclude that Langerin$^+$ cells might be involved in CD pathogenesis, taking into account the fact that interleukin (IL)-15, a central cytokine in the CD mucosa[24,25], can skew DC precursors

Table 1 Numbers, mean ages, genders and IgA-tTG positivity results of the persons studied *n* (%)

Study group	Number of persons	Age[1] (yr)	Male		Female		IgA-tTG-positive
			n	Age[1] (yr)	*n*	Age[1] (yr)	(> 10 U/mL)
CD	18	9.9 ± 10.7	5 (28)[b]	8.3 ± 3.1	13 (72)[b]	10.5 ± 12.5	18/18[b]
CD with T1D	15	8.5 ± 3.7	8 (53)	8.6 ± 4.8	7 (47)	8.5 ± 2.1	11/11
T1D	2	8.5 ± 6.4	2 (100)	8.5 ± 6.4	0	-	0/2
Control group	39	12.8 ± 4.9	14 (36)[b]	11.4 ± 5.9	25 (64)[b]	13.5 ± 4.3	2/35[b]
Total	74	11.1 ± 6.8	29 (39)[b]	9.9 ± 5.2	45 (61)[b]	11.9 ± 7.6	31/66

[1]Value is the mean ± SD. [b]$P \leqslant 0.01$, study group *vs* control group. CD: Celiac disease; T1D: Type 1 diabetes; IgA-tTG: IgA antibody.

to differentiate into Langerin$^+$ cells[26]. In addition, we do not know how Langerin$^+$ DCs are related to other DC subsets and Tregs in the human small intestinal mucosa. Because Langerin$^+$ DCs are significant modulators of events in the skin, another important immunological barrier of the organism, knowledge of the function of these cells in the small intestinal mucosa may be of general importance.

In the present study, we aimed to investigate the densities of CD11c$^+$ DCs, CD103$^+$ DCs, IDO$^+$ DCs and Langerin$^+$ DCs, along with FOXP3$^+$ Treg cells, in the small bowel mucosa in CD patients with and without coexisting T1D and to compare these densities with the those found in histologically normal intestinal mucosa in persons with functional dyspepsia using immunohistochemical and immunofluorescence methods.

MATERIALS AND METHODS

Study population

Seventy-four patients (45 female, 29 male, mean age 11.1 ± 6.8 years) who were admitted to the Children's Clinic of Tartu University Hospital and underwent small bowel biopsy were studied. All patients were recruited at the time of CD diagnosis. CD without T1D was diagnosed in 18 (mean age 9.9 ± 10.7 years) patients, and CD with T1D was diagnosed in 15 (mean age 8.5 ± 3.7 years) patients. Normal small bowel mucosa was found in two T1D patients with equivocal values of IgA antibodies to tissue transglutaminase (tTG, 7.0 and 9.8 U/mL, respectively; both boys, 13 and 4 years old). Because healthy persons could not be included in the control group due to ethical constraints (gastroduodenoscopy), we selected thirty-nine patients (mean age 12.8 ± 4.9 years) with other diagnoses (mainly with functional dyspepsia, duodenal ulcer and erosive gastritis) for the control group (Table 1). Diagnosis of CD was established on the basis of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria[27]. Morphologically, the small bowel mucosa was assessed according to the Marsh classification[28] on the basis of biopsy samples taken by gastroduodenoscopy from distal duodenum.

According to this classification, all CD patients had partial or subtotal villous atrophy: a Marsh grade of IIIa was observed in 9 cases, grade IIIb in 21 cases and grade IIIc in 3 cases. Thirty-nine patients without CD and 2 with T1D had normal small bowel mucosa (Marsh grade 0).

All patients with CD had IgA antibodies to tTG as assessed using an EliA™ Celikey® IgA assay (Pharmacia Diagnostics, Freiburg, Germany) (mean value 414.5 ± 130.5 U/mL). In the control group, two persons with normal small bowel mucosa (a 16-year-old boy and an 8-year-old girl, both with functional dyspepsia) were positive for IgA antibodies to tTG (515.0 and 58.3 U/mL, respectively). The mean value of IgA antibodies to tTG in the control group was 17.9 ± 14.7 U/mL.

Ethics

This study complies with the Declaration of Helsinki and was approved by the Ethics Review Committee for Human Research of the University of Tartu. All studied children and/or their parents gave written informed consent to participate in the study.

Material

Small bowel biopsy from the distal duodenum was performed by gastroduodenoscopy. Two specimens were used for morphological and immunohistochemical examinations, and the third specimen was immediately quick-frozen in Tissue Tek OCT Compound (Sakura, Finetek, Finland) and stored at -80 ℃ for further use in immunofluorescence studies.

Immunohistochemistry on paraffin-embedded specimens

The following antibodies were used: monoclonal mouse anti-CD11c (NCL-L-CD11c-563) Novocastra™ Liquid, diluted 1:80; monoclonal mouse anti-human FOXP3 antibodies (clone 236A/E7, Abcam, Cambridge, United States), diluted 1:60 (16.6 μg/mL in 1% normal horse serum); and anti-IDO (mouse monoclonal-anti-human indoleamine 2,3-dioxygenase), clone 1F8.2 (Chemicon, Millipore Corporation), diluted 1:50.

For the detection of CD11c$^+$ DCs and FOXP3$^+$ T cells in the small bowel mucosa of 63 patients, double staining was used; mono-staining for IDO$^+$ DCs was performed in 58 patients.

Formalin-fixed biopsy specimens of the small bowel mucosa were studied using the Avidin-Biotin method. Paraffin slides were deparaffinized, and antigen retrieval was achieved by microwave treatment in 1 mmol/L

EDTA (Scharlau Chemie S.A., pH 8.0), once at 900 W for 7 min and twice at 440 W for 5 min. After cooling for 20 min at room temperature (rt), endogenous peroxidase activity was quenched by incubating the slides for 30 min at rt in 0.5% H_2O_2-methanol. To avoid nonspecific reactions, slides were treated with 2.5% normal horse serum (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, United States) for 10 min at rt. Additionally, to block the binding of antibodies to the Fc receptor, the FcR Blocking Reagent (human, Miltenyi Biotec GmbH, Germany), diluted 1:100, was used for 10 min at 4 ℃. The sections were incubated with monoclonal mouse anti-CD11c antibody for 15 min at rt, then overnight at 4 ℃. We used biotinylated anti-mouse IgG (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, United States), diluted 1:200 (incubation for 30 min at rt), as the secondary antibody. The bound antibody was detected with a commercial avidin-biotin immunoperoxidase system (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, United States) according to the manufacturer's instructions, using the Vector VIP Peroxidase substrate kit (SK-4600) (incubation for 10 min) (purple-red staining). The reaction was stopped by rinsing the sections in cold water, after which staining for FOXP3 was performed using monoclonal mouse anti-human FOXP3 antibodies for 1 h at rt, followed by incubation overnight at 4 ℃. After washing in 1 × Tris-buffered saline (Tris-HCI, pH 7.5), the sections were incubated with the above-mentioned secondary antibodies using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, United States). The bound antibody was visualized using Vector SG (Vector Laboratories, Burlingame, CA, United States) as the substrate (incubation for 10 min) (blue staining). Next, the sections were immersed in cold water to stop the reaction, after which the stained tissue sections were dehydrated and mounted in Canada balsam. We used tissue sections without primary antibody (incubation with 1% horse serum) for the negative control and human tonsil sections for the positive control.

Staining for IDO^+ DCs on paraffin sections was performed as described above; primary antibodies were incubated with the sections overnight at 4 ℃. The bound antibody was detected with a commercial avidin-biotin immunoperoxidase system (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, United States) according to the manufacturer's instructions, using the Vector VIP Peroxidase substrate kit (SK-4600) (incubation for 10 min) (purple-red staining).

Immunofluorescence staining on cryostat sections

Double-staining for IDO and CD103 and for CD11c and CD103 was performed on frozen sections of the small bowel mucosa of 71 patients. Mono-staining for Langerin (CD207) on sections from the same frozen biopsies was performed for 70 patients. The 4 μm frozen sections cut with a cryomicrotome (Leica CM1950, Leica Microsystems, Germany) were mounted on SuperFrost Plus slides (Menzel GmbH and Co KG, Braunschweig, Germany), air-dried for 1 h and used immediately. Tissue sections were fixed in 4% paraformaldehyde for 10 min at rt, followed by permeabilization with 0.3% Triton-X 100 (SERVA, Feinbiochemica, Heidelberg, Germany) in Tris buffer for 30 min at rt.

Before incubation with primary anti-IDO antibodies, endogenous biotin was blocked using the Biotin Blocking System Kit (DAKO, Cytomation, Carpinteria, United States) for 10 min at rt. Additionally, to block the binding of antibodies to the Fc receptor, the FcR Blocking Reagent (human from Miltenyi Biotec GmbH, Germany), diluted 1:100, was used on all studied sections for 10 min at 4 ℃. This reagent was also used before incubation with anti-CD11c, anti-CD103 and anti-Langerin primary antibodies.

The sections were incubated with anti-IDO clone 1F8.2 (Chemicon, Millipore Corporation) monoclonal mouse-anti human IDO antibody, diluted 1:50, in TRIS buffer overnight at 4 ℃ in a humid chamber under coverslips. For the secondary antibody, we used biotinylated anti-mouse IgG (in horse), diluted 1:150 (Vector Laboratories, Burlingame, CA, United States), and a positive reaction was visualized after the incubation with Streptavidin-Alexa blue 350 (Invitrogen, by Life Technologies), diluted 1:100, for 1 h at rt. After that, the slides were double-stained with anti-CD103 [Integrin αE (N-19): sc-6606, Santa Cruz Biotechnology] polyclonal goat-anti-human antibody, diluted 1:50, overnight at 4 ℃, followed by incubation with donkey-anti-goat-Alexa 488 (Invitrogen, by Life Technologies), diluted 1:100 and incubated for one hour at rt, as the secondary antibody.

Cryostat sections cut from the same frozen biopsies were incubated in parallel with anti-CD11c [Integrin alpha X (H-68): sc-28663, Santa Cruz Biotechnology] and polyclonal rabbit-anti-human (diluted 1:50 and incubated overnight at 4 ℃), followed by incubation with anti-rabbit IgG (whole molecule) conjugated with a Cy3 (Fab') fragment sheep antibody (SIGMA-Aldrich, United States), diluted 1:100 and incubated for one hour at rt. After washing in TRIS buffer, the slides were double-stained with anti-CD103 [Integrin αE (N-19): sc-6606. Santa Cruz Biotechnology] polyclonal goat-anti-human (diluted 1:50 and incubated overnight at 4 ℃), followed by incubation with donkey-anti-goat-Alexa 488 (Invitrogen, by Life Technologies), diluted 1:100 and incubated for one hour at rt, as the secondary antibody.

A separate cryostat section from the same frozen biopsy specimen was incubated with anti-Langerin (CD207) (N-14) goat-anti-human polyclonal antibody (Santa Cruz Biotechnology), diluted 1:50 and incubated overnight at 4 ℃, followed by incubation with donkey-anti-Goat-Alexa 488 (Invitrogen, by Life Technologies), diluted 1:100 and incubated for one hour at rt, as the secondary antibody. After washing in TRIS buffer, the slides were mounted in TRIS-glycerol solution. The negative control was performed by omitting the primary antibody (incubation with TRIS buffer alone).

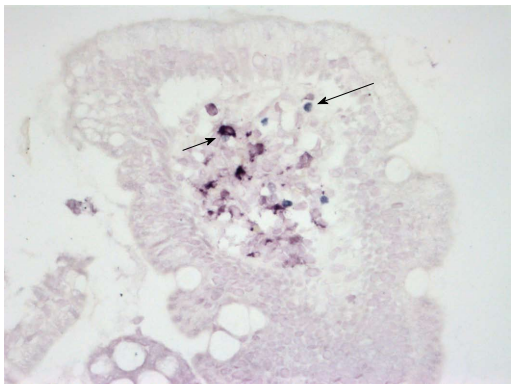

Figure 1 Double staining for CD11c (purple-red, short arrow) and FOXP3 (blue, long arrow) on a paraffin section of the small bowel mucosa. Original magnification, objective × 40; eyepiece × 10.

The paraffin sections were microscopically examined using a objective × 40 and a eyepiece × 10 in a Zeiss KF 2 transmitted light microscope (Carl Zeiss, Jena, Germany). The cryosections were examined under an immunofluorescence microscope (Olympus BX50, Japan) using objectives with magnification × 40 and an eyepiece with magnification × 10. The micro-photos were obtained using a Leica DM5500 B (Leica Microsystems CMS GmbH, Germany) under the same magnifications.

All sections were studied with the investigator blinded to the diagnosis data. In each of the 5 different microscopic fields, DCs positive for CD11c, CD103, IDO and Langerin, along with lymphocytes positive for FOXP3, were counted. Cell densities were expressed as the mean number of positively stained cells per field. The results of double-staining for IDO and CD11c and for CD11c and CD103 were analyzed in a similar manner.

Tissue transglutaminase IgA antibody immunoassay using the EliA™ Celikey® IgA assay.

Sixty-six sera samples were tested for IgA antibody (IgA-tTG) using a fully automated EliA™ Celikey® IgA assay (Pharmacia Diagnostics, Freiburg, Germany) according to the manufacturer's instructions. According to the manufacturer's suggestions, IgA-tTG values higher than 10 EliA U/mL were considered positive.

Statistical analysis

The results obtained for the different study groups are presented as mean ± SE. Statistical calculations were performed using the Graph Pad Prism 5.0 software using the *t*-test, Mann-Whitney *U* test and Spearman's rank correlation test. The χ^2 or Fisher exact test was used for nominal variables.

Differences were considered statistically significant at $P < 0.05$. Sensitivity, specificity and receiver operating characteristic (ROC) curves with the areas under the curve (AUC) were calculated for different DC markers and FOXP3 using StatsDirect software.

RESULTS

Using double-staining on paraffin sections, both $CD11c^+$ DCs and $FOXP3^+$ Treg cells were detected in the small bowel mucosa (Figure 1).

The density of $CD11c^+$ DCs was significantly increased in CD patients compared with patients with normal mucosa ($P = 0.007$) (Figure 2A). The numbers of $FOXP3^+$ Tregs were significantly higher in CD patients ($P = 0.0002$) and in patients with CD and coexisting T1D ($P = 0.002$) compared with patients with normal mucosa (Figure 2B). The density of $FOXP3^+$ Tregs significantly correlated with the histological grade of atrophic changes in the small bowel mucosa according to the March classification ($r = 0.62$; $P < 0.0001$) and with levels of IgA-tTG ($r = 0.55$; $P < 0.0001$) (Figure 2C and D).

IDO^+ DCs were detected on paraffin sections by mono-staining using the avidin-biotin immunohistochemical method (Figure 3).

The densities of IDO^+ DCs were significantly higher in CD patients ($P = 0.00003$) and in patients with CD and coexisting T1D ($P = 0.004$) compared with patients with normal mucosa (Figure 4A). This difference was dependent on the grade of atrophic and inflammatory changes in the small bowel mucosa, *e.g.*, in subtotal villous atrophy (grade IIIb, according to Marsh), the mean value of IDO^+ DCs was 10.37 ± 1.24 *vs* 6.40 ± 1.75 in partial villous atrophy (grade IIIa; $P = 0.03$). This finding is also supported by analysis of the correlation of density of IDO^+ DCs on paraffin sections with the histological grade of the small bowel mucosa ($r = 0.64$; $P < 0.0001$) and with levels of IgA-tTG ($r = 0.48$; $P = 0.0002$) (Figure 4B and C). A significant correlation was established between the densities of IDO^+ DCs and $FOXP3^+$ Treg cells on paraffin sections for the entire study group ($r = 0.76$; $P = 0.0001$) (Figure 4D).

To simultaneously study the densities of $CD11c^+$, $CD103^+$ and IDO^+ DCs in the same tissue section, we used double immunofluorescence staining on two consecutive cryostat serial sections from the same biopsy specimen of the small bowel mucosa - one for CD103 and IDO (Figure 5A) and the other for CD103 and CD11c (Figure 5B). The mean values of $CD103^+$ DCs were significantly higher in CD patients ($P = 0.01$) and in patients with CD and associated T1D ($P = 0.00002$) compared with subjects with normal small bowel mucosa (Figure 6A). The density of $CD103^+$ DCs was correlated with the histological grade of small bowel mucosa atrophy ($r = 0.39$; $P = 0.0008$) (Figure 6B). A significant correlation was identified between the densities of $CD103^+$ DCs and $FOXP3^+$ Tregs for the entire study group ($r = 0.33$; $P = 0.0087$) (Figure 6C). The mean values of $CD11c^+$, IDO^+ and $CD103^+$ DCs were significantly higher in CD patients compared with persons with normal small bowel mucosa (Figure 7).

The IDO^+ and $CD103^+$ markers were simultaneously expressed in 30% of the DCs in the LP of the bowel

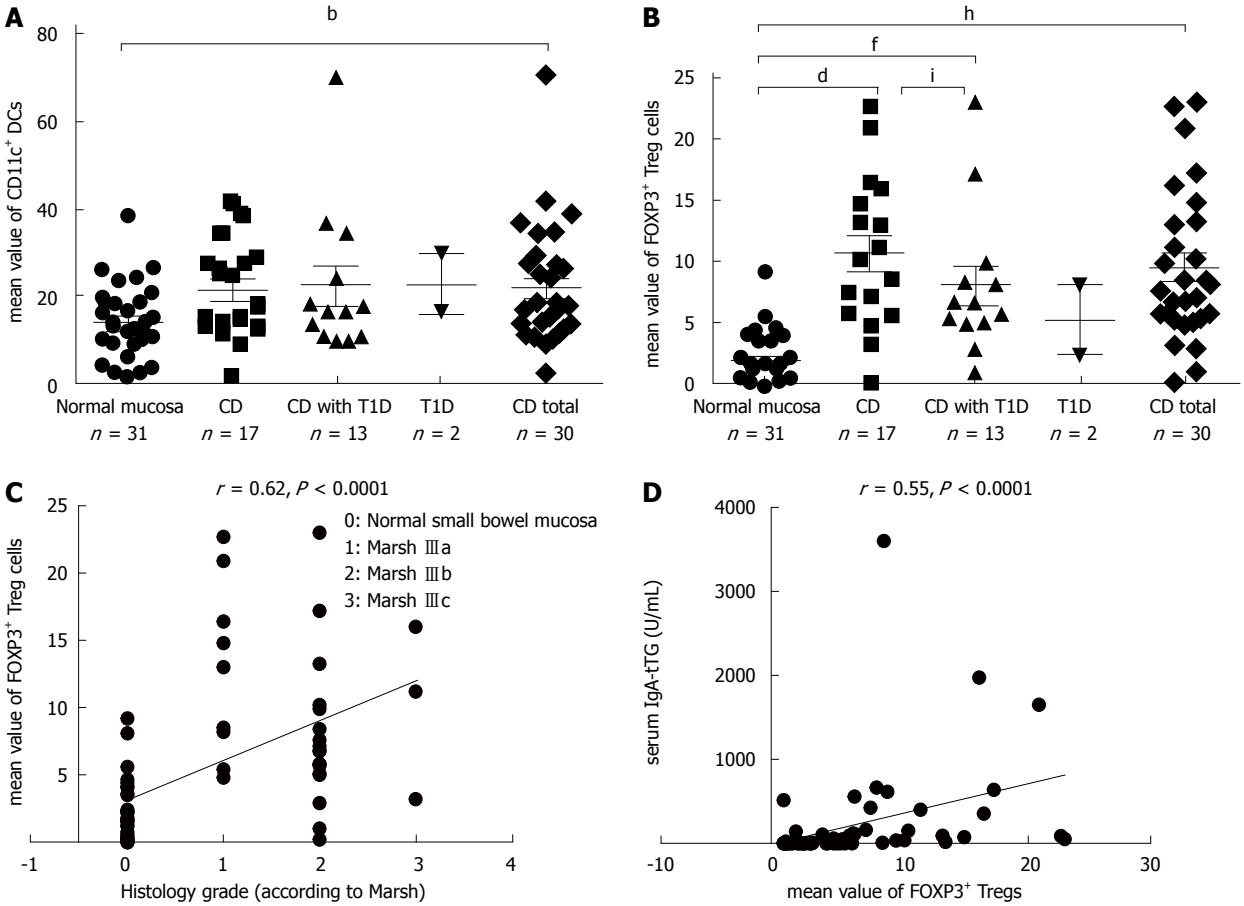

Figure 2 Density of CD11c$^+$ dendritic cells (A), FOXP3$^+$ regulatory T cells (B) in paraffin sections for the different study groups; Spearman's rank correlation between the histological grades (according to Marsh) and FOXP3$^+$ regulatory T cells cell densities (C) or between the densities of FOXP3$^+$ regulatory T cells and serum IgA-tTG levels (D) in the studied persons. $^bP < 0.01$, CD total *vs* normal mucosa; $^dP < 0.01$, CD *vs* normal mucosa; $^fP < 0.01$, CD with T1D *vs* normal mucosa; $^hP < 0.01$, CD total *vs* normal mucosa; ^iP value for CD *vs* CD with T1D not statistically significant. CD: Celiac disease; T1D: Type 1 diabetes; DC: Dendritic cell; IgA-tTG: IgA antibody; Tregs: regulatory T cells.

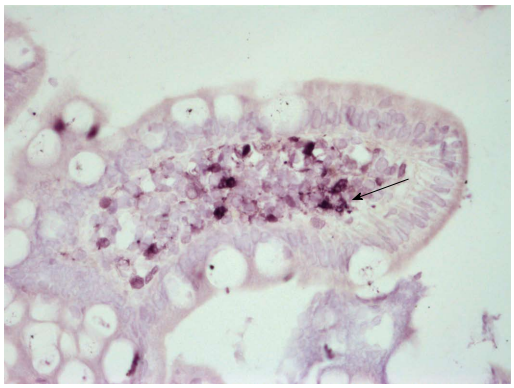

Figure 3 Positive staining for indoleamine 2,3-dioxygenase (purple-red) on a paraffin section of the small bowel mucosa. Original magnification, objective × 40; eyepiece × 10.

mucosa of CD patients and in 25% of the DCs in the normal mucosa. Both markers, CD11c and CD103, were expressed in 5% of the DCs in the LP of the bowel mucosa of the studied persons, with CD11c accounting for 47.5% and CD103 accounting for 47%. Double expression of CD11c and CD103 was observed in 6.5% of the DCs in the CD group compared with 4.2% of the visualized DCs in persons with normal mucosa.

Immunofluorescence staining for Langerin on a cryostat section of the small bowel mucosa is presented in Figure 8. Langerin$^+$ DCs were identified in both the LP (Figure 8A) and the epithelium of the villus of the small bowel mucosa (Figure 8B).

The mean value of Langerin$^+$ DCs was higher in CD patients compared with persons with normal mucosa ($P = 0.04$; Figures 7 and 9). The correlations between Langerin$^+$ DCs and CD11c$^+$ DCs ($r = 0.57$; $P = 0.04$) and between Langerin$^+$ DCs and CD103$^+$ DCs were more pronounced in female CD patients ($r = 0.77$; $P = 0.002$).

According to the analysis of the ROC curves, FOXP3 and IDO positivity had the highest discriminative power (AUC) for both CD (AUC = 0.92 for FOXP3 and 0.88 for IDO) and CD with coexisting T1D (AUC = 0.91 for FOXP3 and 0.91 for IDO), while CD103 had also a high discriminative power for CD associated with T1D (AUC = 0.86) (Table 2 and Figure 10).

The densities of IDO$^+$ DCs (6.55 ± 0.51 *vs* 4.67 + 0.72), CD103$^+$ (7.16 ± 0.85 *vs* 4.93 ± 0.65) and CD11c$^+$ DCs (7.62 ± 0.43 *vs* 5.83 + 0.37) were significantly higher in females compared with males ($P = 0.042$, $P = 0.04$ and $P = 0.003$, respectively). The number of Langerin$^+$ DCs

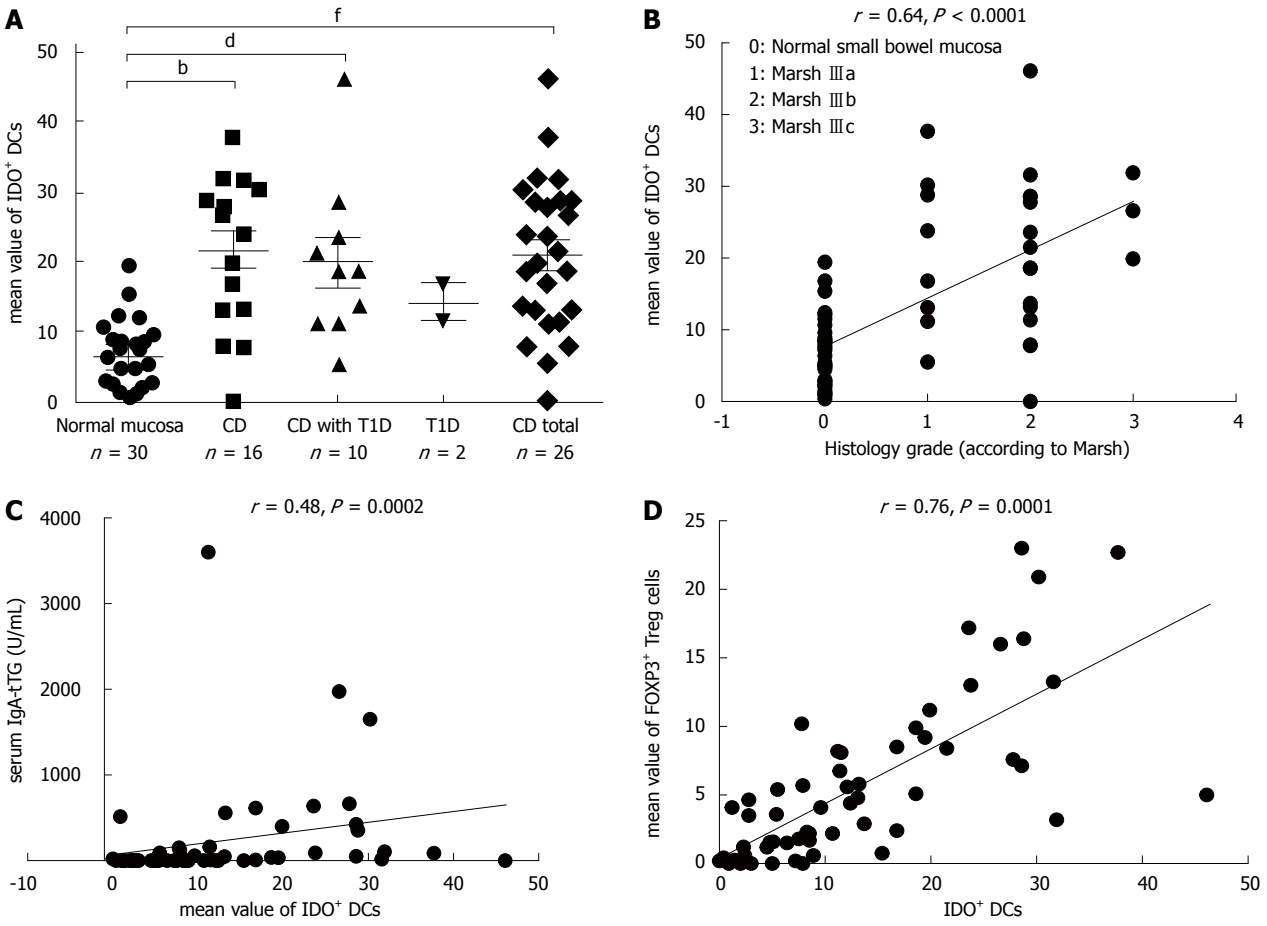

Figure 4 Densities of IDO$^+$ DCs on paraffin sections for the different study groups (A); Spearman's rank correlation between the histological grades (according to Marsh) and IDO$^+$ DCs (on paraffin sections) for the studied persons (B); between the densities of IDO$^+$ DCs and serum IgA-tTG levels in the studied persons (C); or between the densities of IDO$^+$ DCs and FOXP3$^+$ Treg cells for the entire study group, evaluated on paraffin sections (r = 0.76; P = 0.0001) (D). The dots represent the mean values of positively stained cells per microscopic field. $^bP < 0.01$, CD *vs* normal mucosa; $^dP < 0.01$, CD with T1D *vs* normal mucosa; $^fP < 0.01$, CD total *vs* normal mucosa. CD: Celiac disease; DC: Dendritic cell; T1D: Type 1 diabetes; IgA-tTG: IgA antibody; Tregs: regulatory T cells; IDO: Indoleamine 2,3-dioxygenase.

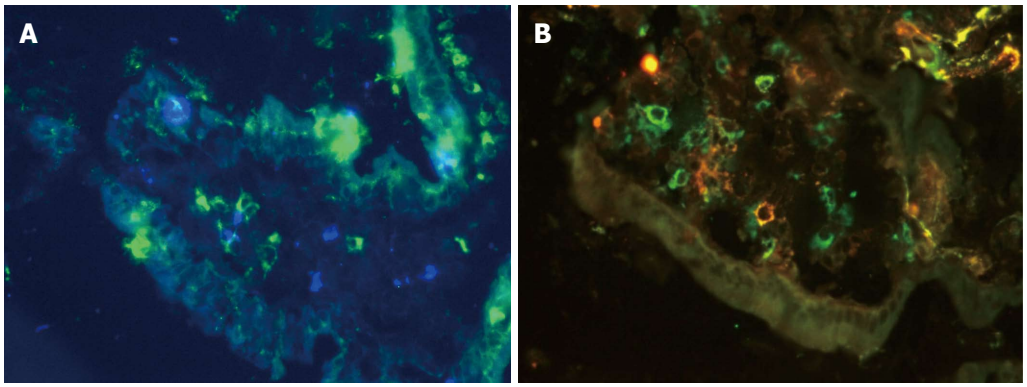

Figure 5 Double-staining for IDO (blue) and CD103 (green) (merged, A) and double-staining for CD11c (red) and CD103 (green) (merged, B) on cryostat sections of the small bowel mucosa. Original magnification, objective × 40; eyepiece × 10. IDO: Indoleamine 2,3-dioxygenase.

(6.27 ± 0.61 *vs* 4.56 ± 0.62) was also higher in females than in males (P = 0.01). Among all persons studied, the density of IDO$^+$ DCs was significantly higher in females compared with males (8.41 ± 0.70 and 5.41 ± 0.73, respectively; P = 0.004).

DISCUSSION

The main finding of this study was the significantly higher densities of CD11c$^+$, tolerogenic IDO$^+$, CD103$^+$ and Langerin$^+$ DCs in the small bowel mucosa of

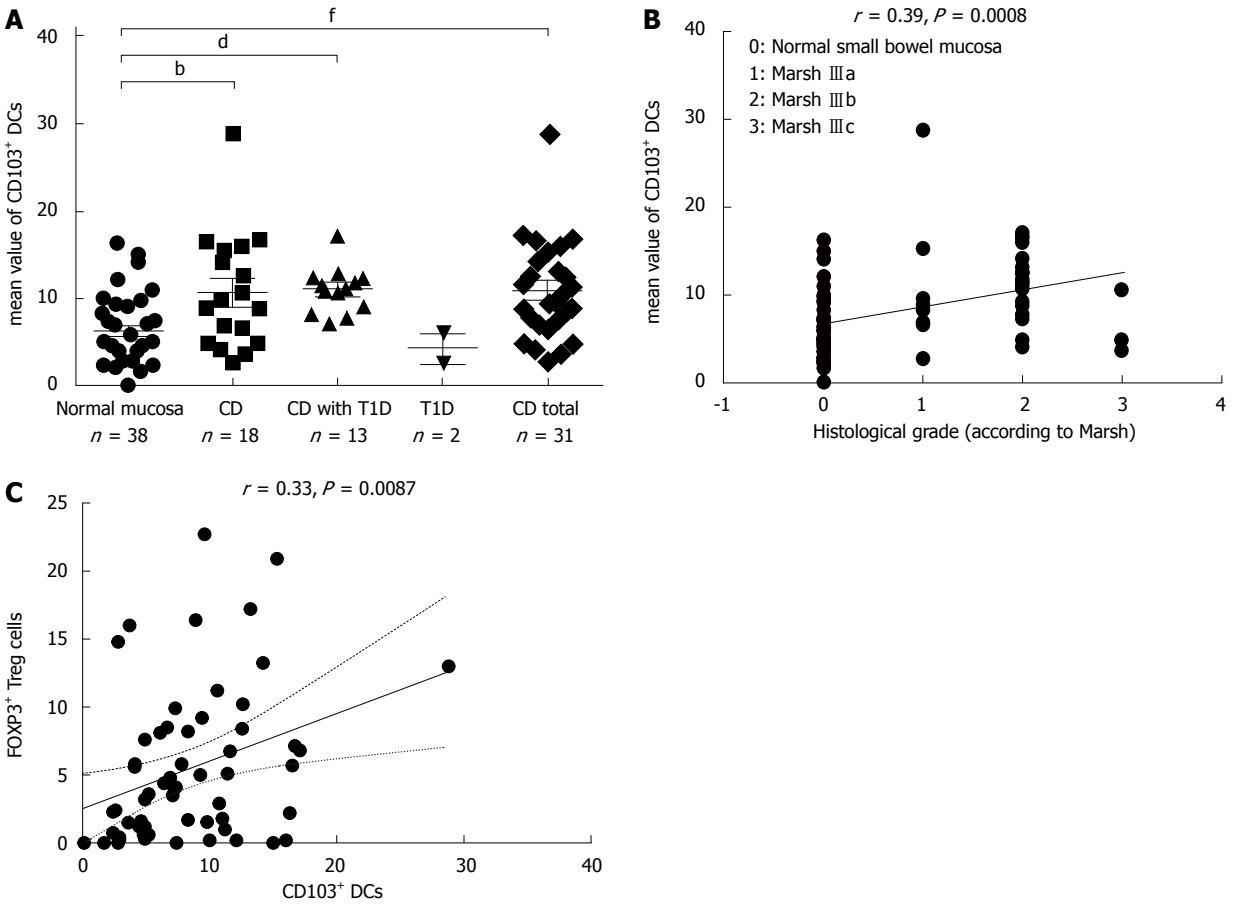

Figure 6 Densities of CD103$^+$ DCs on cryostat sections for the different study groups (A); Spearman's rank correlation between the histological grades (according to Marsh) and the densities of CD103$^+$ DCs for the studied persons (B); or between the densities of CD103$^+$ DCs and FOXP3$^+$ Treg cells for the entire study group (C). The dots represent the mean values of positively stained cells per microscopic field. $^bP \leq 0.01$, CD *vs* normal mucosa; $^dP < 0.01$, CD with T1D *vs* normal mucosa; $^fP < 0.01$, CD total *vs* normal mucosa. CD: Celiac disease; T1D: Type 1 diabetes; DC: Dendritic cell; Tregs: regulatory T cells..

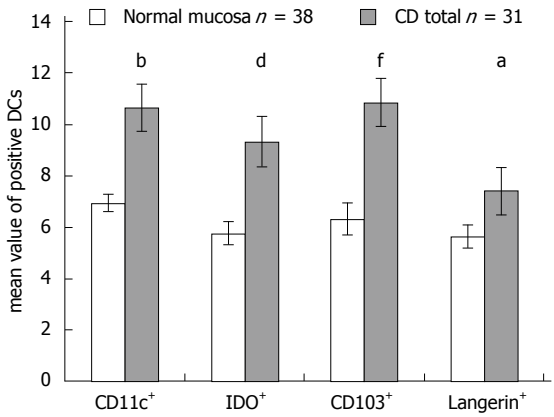

Figure 7 Mean values of CD11c$^+$, IDO$^+$, CD103$^+$ and Langerin$^+$ DCs in celiac disease patients are significantly higher compared with these values in persons with normal small bowel mucosa (according to the immunofluorescence data from cryostat sections). Columns represent the mean values of positively stained cells per microscopic field in patients with CD *vs* patients with normal mucosa. $^bP < 0.01$, CD11c$^+$ in patients with CD *vs* patients with normal mucosa; $^dP < 0.01$, IDO$^+$ in patients with CD *vs* patients with normal mucosa; $^fP < 0.01$, CD103$^+$ in patients with CD *vs* patients with normal mucosa; $^aP < 0.05$, Langerin$^+$ CD103$^+$ in patients with CD *vs* patients with normal mucosa. CD: Celiac disease; IDO: Indoleamine 2,3-dioxygenase; DC: Dendritic cell.

patients with CD compared with subjects with normal small bowel mucosa. The densities of IDO$^+$ and particularly of CD103$^+$ DCs were high in CD patients with coexisting T1D, possibly demonstrating the strongest pressure on the local immune system in these patients. These results are consistent with our finding that FOXP3$^+$ Tregs are present at higher densities in CD patients with and without coexisting T1D. A significant correlation between the densities of tolerogenic DCs and FOXP3$^+$ Tregs in the small bowel mucosa of the persons studied might confirm the involvement of these DCs in enhanced FOXP3$^+$ Tregs development.

Accumulation of CD11c$^+$ DCs in celiac lesions was also observed in a study by Ráki *et al*[29], who showed that CD11c$^+$ DCs are able to activate gluten-reactive T cells. In a subsequent study, the same group of authors established that rapid accumulation of CD14$^+$CD11c$^+$ DCs occurred in the LP of the gut mucosa of treated patients with CD after a three-day gluten challenge and asserted that gluten-induced recruitment of these cells is specific for CD[30]. Another study by this group demonstrated the increased density of CD163$^+$CD11c$^+$ DCs in celiac lesions[31]. However, according to their

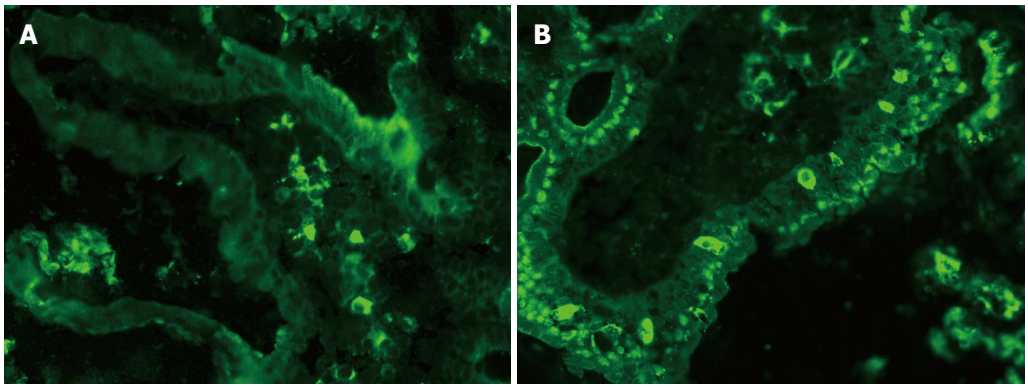

Figure 8 Staining for Langerin (CD207) on cryostat sections of the small bowel mucosa in the lamina propria (A) and in the epithelium of the villus of the small bowel mucosa (B). Original magnification, objective × 40; eyepiece × 10.

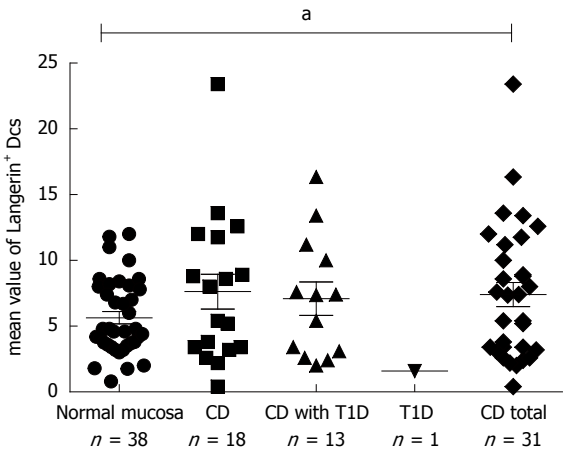

Figure 9 Densities of Langerin$^+$ dendritic cells on paraffin sections for the different study groups. The dots represent the mean values of positively stained cells per microscopic field. $^aP < 0.05$, CD total *vs* normal mucosa. CD: Celiac disease; T1D: Type 1 diabetes; DC: Dendritic cell.

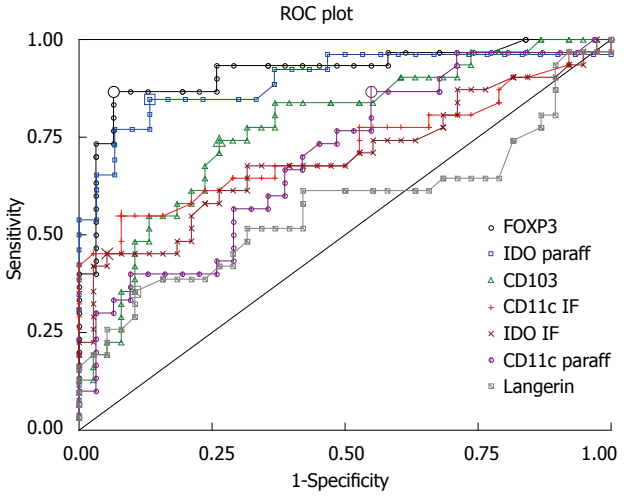

Figure 10 Receiver operating characteristic curves of dendritic cell and Tregs markers, showing their overall capacity to discriminate between individuals with and without the disease. The diagonal line represents a worthless test (sensitivity = 1 - specificity). The further the curve is from the diagonal line and the closer it is to the upper left- hand corner of the graph, the better the discriminative power of the test. CD: Celiac disease; IDO: Indoleamine 2,3-dioxygenase; ROC: Receiver operating characteristic.

results, the density of CD103$^+$ DCs was decreased in CD patients. Importantly, in these studies, the study group consisted of older patients (mean age 39 years) compared with the patients in our CD group (mean age 11.1 ± 6.8 years), which might at least partly explain the discrepancies between the results of their study and ours. Thus, we believe that the increased density of CD103$^+$ DCs in the proximal part of the small intestinal mucosa of CD patients, particularly those with coexisting T1D, is of pathogenic relevance.

Another important finding in our study was the significant correlation between densities of IDO$^+$ DCs and FOXP3$^+$ Tregs, indicating the tolerogenic capacity of IDO$^+$ DCs. Moreover, 29.7% of the LP DCs were double-positive for both IDO and CD103 markers in the CD patient group. Thus, our data are in agreement with those of Matteoli *et al*[18], who reported that IDO expression was particularly associated with CD11c$^+$ CD103$^+$ DCs, both in mouse and human LP. According to these authors, IDO is involved in the capacity of CD103$^+$ DCs to drive FOXP3$^+$ Tregs development.

However, the study of Badami *et al*[32] showed that T1D patients had a reduced number of FOXP3$^+$ Treg cells due to both impaired differentiation by gut-associated CD103$^+$CD11c$^+$ DC and their need to maintain immune tolerance for pancreatic cells. These authors did not find any difference in the amount of intestinal CD103$^+$CD11c DCs in the LP between T1D patients, CD patients and controls. The discrepancy between the results in the above-mentioned study and ours could be partly explained by the different methods used in these two studies. Badami *et al*[32] used multiparametric fluorescent-activated sorter analysis of the cell subsets in the biopsy specimens of the studied persons and a conversion assay for the assessment of tolerogenic function of the LP DCs or blood monocyte-derived DCs *in vitro*. We performed immunohistochemical evaluations of a subpopulation of DCs and T cells in duodenal biopsy specimens. It should also be noted that their T1D group was significantly older (mean age 29

Table 2 Areas under the curve values and the cut-off, sensitivity and specificity values for different dendritic cells and Tregs markers in the groups studied

Markers	Entire study group				Patients with CD				Patients with CD and T1D			
	AUC	Cut-off	Sensitivity	Specificity	AUC	Cut-off	Sensitivity	Specificity	AUC	Cut-off	Sensitivity	Specificity
FOXP3 (paraff)	0.92	4.8	0.87	0.94	0.92	4.8	0.88	0.94	0.91	5.0	0.85	0.94
IDO (paraff)	0.89	11.2	0.85	0.87	0.88	13.1	0.81	0.93	0.91	11.2	0.90	0.87
CD11c (IF)	0.72	10.1	0.55	0.92	0.79	10.1	0.61	0.92	0.62	12.1	0.38	1.00
IDO (IF)	0.70	9.7	0.45	0.95	0.72	10.1	0.50	0.97	0.67	9.7	0.39	0.95
CD11c (paraff)	0.70	10.6	0.87	0.45	0.71	24.8	0.47	0.90	0.68	16.6	0.62	0.71
CD103 (IF)	0.77	7.8	0.74	0.74	0.71	8.8	0.61	0.76	0.86	7.3	1.00	0.68
Langerin (IF)	0.57	8.8	0.36	0.90	0.59	8.6	0.44	0.84	0.55	7.4	0.54	0.68

AUC: Area under the receiver operating characteristic curve; Cut-off: Marker value at which sensitivity and specificity are optimal; CD: Celiac disease; paraff: Immunohistochemistry on paraffin-embedded specimens; IF: Immunofluorescence staining on cryostat sections; T1D: Type 1 diabetes.

years) than our patient and control populations, which might also have influenced the results.

Similarly to other authors, we observed increased expression levels of *FOXP3* mRNA and protein in the small bowel mucosa of patients with CD with and without associated T1D in our previous study, which might indicate an imbalance between regulatory and effector mechanisms in the pathogenesis of these diseases[33,34].

The results of the present study regarding the higher density of FOXP3$^+$ Tregs in CD patients with and without coexisting T1D are consistent with the relevant results of other studies. Cianci *et al*[35] reported increased Tregs in the peripheral blood and the duodenal mucosa of patients with active CD; they regarded this phenomenon as an example of an "immunological niche", where naive T cells recruited by the gluten trigger from the peripheral blood to local mucosa tissue will differentiate into Tregs in the presence of anti-inflammatory cytokines, such as transforming growth factor (TGF)-beta and IL-10. A significant increase in the density of FOXP3$^+$ T regs in the LP of CD patients, correlated with both the histological Marsh grade and the serum levels of transglutaminase type 2 autoantibodies, was demonstrated by Brazowski *et al*[36]. The increased expression of CD4$^+$CD25$^+$FOXP3$^+$ circulating Tregs in untreated CD patients can be explained as an attempt to quench intestinal inflammation and the immune response to dietary gluten[37]. Borrelli *et al*[38] reported an increased density of FOXP3$^+$ Tregs in the duodenal mucosa and in the peripheral blood of patients with potential CD and interpreted this phenomenon as an effort by the immune system to down-regulate current inflammation and to restrict its progression toward mucosal damage through either the redistribution of FOXP3$^+$ Tregs or their local proliferation.

In a study by Kivling *et al*[39], children with CD with or without associated T1D had significantly higher *FOXP3* mRNA expression levels compared with children with only T1D. This difference could indicate increased Tregs-associated activity in the case of two autoimmune disorders (CD and coexisting T1D) in contrast to a single disorder of the immune system (children with only T1D). However, some studies have established impairment of the regulatory activity of both intestinal and peripheral blood FOXP3$^+$ Treg cells in patients with active CD[40-42]. This phenomenon could be explained by the overproduction of IL-15, a cytokine preventing the response of effector T cells to the suppressive effects of Tregs, and partly by the overexpression of IL-15Rα in CD patients[41,43].

There is evidence that IDO plays an important role as a suppressor of lymphocyte-mediated inflammatory responses[17]. In our study, the number of IDO$^+$ DCs was greater in the small bowel mucosa of patients with higher-grade CD, *i.e.*, grade Ⅲb according to the Marsh classification, compared with patients with grade Ⅲa small bowel mucosa. The high discriminative power of the IDO marker in CD patients with and without T1D established in our study also supports the above statement. This finding is in good agreement with the results of Torres *et al*[19], who revealed high expression levels of IDO in intestinal biopsies of CD patients. Moreover, these authors observed that increased levels of interferon-gamma and tumor necrosis factor-alpha were potent inducers of IDO expression levels in CD patients. The increase in IDO activity is considered to be an attempt to control chronic antigen stimulation *via* the down-regulation of T cell-mediated autoimmunity[19].

A central novel finding in our study was the presence of variable densities of Langerin$^+$ (CD207$^+$) cells in patients with normal or atrophic small bowel mucosa, pointing to their role among the other DCs of the intestinal mucosa. The presence of Langerin$^+$ cells in the duodenal epithelium (Figure 8) could indicate the possibility that these cells actively participate in the transportation of antigenic material through the epithelial layer, as has been demonstrated earlier for CD11c$^+$ DCs[44]. The presence of different types of DCs in both the LP and the villous epithelium has also been shown by Farache *et al*[45]. Because the density of Langerin$^+$ DCs in the small bowel mucosa was significantly higher in CD patients compared with persons with normal intestinal mucosa, these cells might indeed play a specific role in CD. This difference may be due to the capacity of Langerin$^+$ DCs to take up and route the antigen(s) from the epithelium into the organelles[44]. Rochereau *et*

al[46] confirmed the presence of CD11c$^+$/Langerin$^+$ DCs in mouse Peyer's Patches, located predominantly in the dome region, which supports their function in antigen uptake. However, Langerin$^+$ DCs could be actively recruited to the CD mucosa through their binding to different sugar residues on microorganisms, and some of these interactions might be directly connected to CD development[47]. The binding of Langerin$^+$ DCs to heparin[48], which is reactive to the disease-specific autoantigen tTG, might have an additional impact, namely because the heparin-binding residues of tTG are strong autoantigenic epitopes[49].

One of the limitations of the study is related to a gender imbalance between the study groups, which might have an influence on the results. In female patients, the density of DCs in normal small bowel mucosa was significantly higher compared with male patients. This difference indicates that some results may have been skewed due to divergences in the composition of the study groups. However, Sankaran-Walters *et al*[50] showed that women have higher levels of T cell proliferation and activation and up-regulation in gene expression-related immune functions in the gut microenvironment in the absence of disease, all of which can predispose women to inflammation-associated diseases. Moreover, DC differentiation and function are regulated by the estrogen receptor ligands[51]. Mao *et al*[52] reported that estrogen-dependent CD11c$^+$CD11b Ly6C$^-$ DCs express Langerin (CD207). Our result regarding the significant correlation between the densities of CD11c$^+$ and Langerin$^+$ DCs, particularly in female patients with CD, is in agreement with the finding of the above authors. Xiao *et al*[53] demonstrated that estrogen can induce IDO expression by DCs through suppression of T cell function *via* the IDO pathway. In our study, the higher prevalence of IDO$^+$ DCs in female compared with male patients, especially in the group with normal mucosa and a mean age of 13.5 ± 4.3 years, could have already been affected by the female hormonal status, although the mean age of male subjects with normal mucosa (11.4 ± 5.8) did not differ significantly from female subjects of similar age (P = 0.25). Despite the gender influences, we still believe that differences in the distribution of the DC subsets in the small intestinal mucosa of CD patients and controls are significant.

Another limitation of our study is that we did not know the microbiota status of the small intestines of the studied persons. We agree that the microbiota can strongly influence and regulate the homeostasis of effector immune cells, including the distribution of different subtypes of DCs and Treg cells, along with other immunoregulatory events, in the small bowel mucosa[3,54]. However, this factor is known to be one of the major limitations of other similar studies, unless the intestinal microbiota is studied specifically[55]. Even in cases where it is studied, the interpretation of study results is difficult because the intestinal microbiota is dependent on different nutritional factors[7,8].

In conclusion, we established that CD patients expressed higher densities of CD11c$^+$, IDO$^+$ and CD103$^+$ DCs and Langerin$^+$ DCs in the small bowel mucosa compared with the control persons. The densities of both FOXP3$^+$ Tregs and IDO$^+$ DCs were significantly increased in CD patients with and without coexisting T1D. A significant correlation was identified between the densities of CD103$^+$ DCs and FOXP3$^+$ Tregs and between the densities of IDO$^+$ DCs and FOXP3$^+$ Tregs. This finding highlights the participation of diverse DC subsets in the pathological processes of CD and indicates the possible involvement of tolerogenic DCs in Tregs development to maintain intestinal immunological tolerance in CD patients. Our results demonstrate the diversity of the mechanisms of immunoregulation and the various types of DC involvement in CD, emphasizing the importance of Langerin$^+$ DCs in the small intestinal mucosa.

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COMMENTS

Background

The role of the gut immune system is thought to be of crucial importance in the pathogenesis of celiac disease (CD) and type 1 diabetes (T1D). Several research groups have demonstrated a marked association between the development of T1D and preceding alterations in the small bowel mucosa. Dendritic cells (DCs) have received much attention in both diseases due to their strategic role in gut homeostasis by processing external antigens (including wheat proteins) and by determining tolerance to self-antigens.

Research frontiers

The important role of CD11c$^+$CD103$^+$ DCs and indoleamine 2,3-dioxygenase (IDO) in the induction of regulatory T cells (Tregs) differentiation have been established. In addition, DCs carrying Langerin (CD207) proteins have received considerable interest. The expression of Langerin by CD103$^+$CD11b$^+$ lamina propria DCs in the human ileum has recently been reported. However, the presence of Langerin$^+$ DCs in the small bowel mucosa in pathological conditions such as CD and T1D has not yet been studied. We also do not know how Langerin$^+$ DCs are related to other DC subsets and Tregs in the human small intestinal mucosa. Because Langerin$^+$ DCs are significant modulators of events in the skin, another important immunological barrier of the organism, knowledge of the function of these cells in the small intestinal mucosa may be of general importance. This research aims to investigate the densities of CD11c$^+$ DCs, CD103$^+$ DCs, IDO$^+$ DCs and Langerin$^+$ DCs, along with FOXP3$^+$ Tregs, in the

small bowel mucosa in CD patients with and without coexisting T1D and to compare these densities with those found in histologically normal intestinal mucosa in persons with functional dyspepsia using immunohistochemical and immunofluorescence methods.

Innovations and breakthroughs

The main finding of this study was the significantly higher densities of CD11c$^+$, tolerogenic IDO$^+$, CD103$^+$ and Langerin$^+$ DCs in the small bowel mucosa of patients with CD compared with subjects with normal small bowel mucosa. The densities of IDO$^+$ and particularly of CD103$^+$ DCs were high in CD patients with coexisting T1D, possibly demonstrating the strongest pressure on the local immune system in these patients. A significant correlation between the densities of tolerogenic DCs and FOXP3$^+$ Tregs in the small bowel mucosa of the persons studied might confirm the involvement of these DCs in enhanced FOXP3$^+$ Tregs development.

Applications

This finding highlights the participation of diverse DC subsets in the pathological processes of CD and indicates the possible involvement of tolerogenic DCs in Tregs development to maintain intestinal immunological tolerance in CD patients. This results demonstrate the diversity of the mechanisms of immunoregulation and the various types of DC involvement in CD, emphasizing the importance of Langerin$^+$ DCs in the small intestinal mucosa.

Terminology

IDO is an immunomodulatory enzyme involved in tryptophan catabolism with immunosuppressive effects that has been implicated in the control of intestinal inflammation. Higher IDO expression has been measured in intestinal biopsies from CD patients. Langerin was originally identified as a Langerhans cell -specific C-type lectin receptor involved in antigen capture. Tolerogenic DCs are dendritic cells, predominantly CD103$^+$, that are isolated from lamina propria and mesenteric lymph nodes and are able to drive the development of CD4$^+$FOXP3$^+$ Tregs. Tregs are a subpopulation of CD4$^+$CD25$^+$FOXP3$^+$ T cells that modulate the immune system, mainly by immunosuppressive activity, and play an important role in intestinal homeostasis.

Peer review

The current manuscript studied DCs and FOXP3-positive Tregs and their interactions in the small bowel mucosa of patients with CD with or without T1D.

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ORIGINAL ARTICLE

Basic Study

Aberrant EphB/ephrin-B expression in experimental gastric lesions and tumor cells

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Abstract

AIM: To determine whether the expression profiles of EphB receptor and ephrin-B ligand can be used as markers for dysplastic/oncogenic transformation in gastric mucosa.

METHODS: The protein expression and localization of EphB and ephrin-B in normal, ulcerated regenerating, and dysplastic gastric mucosa were examined in a rat experimental model by immunolabeling, and mRNA expression was assessed in four human gastric carcinoma cell lines by reverse transcription-polymerase chain reaction.

RESULTS: Ephrin-B- and EphB-expressing regions were divided along the pit-gland axis in normal gastric units. EphB2 was transiently upregulated in the experimental ulcer, and its expression domain extended to gastric pits and/or the luminal surface where ephrin-B-expressing pit cells reside. EphB2, B3, and B4 and ephrin-B1 were coexpressed in the experimental gastric dysplasia, and more than one ligand-receptor pair was highly expressed in each of the gastric carcinoma cell lines.

CONCLUSION: Robust and stable coexpression of EphB and ephrin-B is a feature common to experimentally induced gastric dysplasia and human gastric carcinoma cell lines as compared to normal gastric and ulcerated regenerating epithelia. Thus, EphB/ephrin-B may be a useful marker combination for dysplastic/oncogenic transformation in gastric cancer.

Key words: Gastric ulcer; Gastric dysplasia; Gastric carcinoma cell line; Coexpression; EphB; Ephrin-B

Core tip: A constant/high level of EphB and ephrin-B coexpression was identified as a feature common to experimentally induced gastric dysplasia and human gastric carcinoma cell lines, as compared to normal and regenerating gastric epithelia. Based on these, we proposed that the stable/robust EphB and ephrin-B coexpression is a marker of dysplastic/oncogenic transformation. Eph signaling in tumor cells likely has a suppressive role during tumor progression, with Eph and ephrin coexpressed on the same cell engaging in

non-productive interactions *via* lateral inhibition and thereby silencing downstream signaling. These results can be useful for the early and accurate diagnosis of gastric tumors.

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INTRODUCTION

The large Eph receptor tyrosine kinase superfamily has 14 members in mammals that are divided into EphA (A1-A8 and A10) and EphB (B1-B4 and B6; EphB5 has only been detected in the chicken) classes on the basis of sequence homology of the extracellular domain[1,2]. Members of these two receptor classes promiscuously bind ligands of the ephrin-A (A1-A5) and -B (B1-B3) classes, respectively. Ephrin-A members are anchored to the plasma membrane through a glycosyl phosphatidylinositol linkage, while ephrin-B is a class of transmembrane proteins. The Eph/ephrin interaction results in bidirectional signal propagation in both receptor- and ligand-expressing cells. Forward signaling by Eph depends mainly on autophosphorylation by the tyrosine kinase domain and association of the receptor with various effector proteins, while reverse signaling by ephrin depends in part on tyrosine phosphorylation of the cytoplasmic region of ephrin-Bs and associated proteins[1,2]. This cell-cell communication is essential to the development and physiology of various tissues and organs, especially in the nervous and vascular systems[1,3]. Accumulating evidence also implicates Eph/ephrin signaling in tumor development and progression: overexpression, reduced expression, and mutations in the receptor and/or ligand affect tumor cell growth, migration, invasion, and metastasis *in vitro* and *in vivo*[4,5].

The stomach is lined with a simple columnar epithelium on the luminal surface that forms deep tubular invaginations termed gastric glands. These are connected to gastric pits, which are wide tubular depressions on the luminal surface of the mucosa. The isthmus lies between the pits and glands, with the three comprising a gastric unit. Gastric stem cells located in the isthmus proliferate and differentiate to give rise to pit and gastric gland cells; the former migrate apically towards the gastric lumen, whereas the latter migrate basally towards the neck and then to the base of the glands[6-8]. We previously reported complementary expression patterns for EphB and ephrin-B members in rodent gastric mucosa, with receptors and ligands being preferentially expressed in deeper and superficial regions, respectively, of gastric units[9-11]. This finding indicates that EphB/ephrin-B signaling is mostly restricted to the isthmus, where the overlap between receptor and ligand expression domains is highest. We also showed that EphB signaling in primary gastric epithelial cells promoted cell retraction, and proposed that the EphB-positive progeny of gastric stem cells migrates from the isthmus to the bottom of the gastric glands *via* contact-mediated repulsion[11].

Up- or downregulation in expression and mutations in genes of Eph receptors or ephrin ligands have been reported in human gastric tumors[12-25], and EphA overexpression in tumors is correlated with cancer progression, metastasis, and/or poor prognosis[16,18,21-23,25]. Less attention has been given to EphB in gastric tumors. A few studies have shown altered or absent expression of certain receptors in tumor samples relative to adjacent normal tissue, with reduction/loss in expression correlated with gastric cancer progression, metastasis, and poor prognosis[20,24]. In colorectal tumors, the expression of EphB receptors is high during early stages of tumor progression, and downregulated at the adenoma-carcinoma transition[26,27].

Detailed comparisons of the expression profiles of Eph receptors and their ephrin ligands between normal, ulcerated regenerating, and dysplastic gastric epithelia and gastric tumors are lacking. In the present study, expression profiles of EphB/ephrin-B in gastric epithelia were assessed in normal and experimentally induced ulcerated and dysplastic tissues, as well as human gastric carcinoma cell lines, to determine whether EphB/ephrin-B expression can serve as a marker for dysplastic/oncogenic transformation in gastric mucosa.

MATERIALS AND METHODS

Animals

F344 male rats (Japan SLC, Inc., Hamamatsu, Japan) were maintained under standard housing and feeding conditions. Tissue samples from normal and gastric ulcer model rats (8-10 wk old) were used for reverse transcription-polymerase chain reaction (RT-PCR) and immuno- and lectin fluorescence labeling experiments, while immunoperoxidase staining was carried out using samples from gastric dysplasia model rats (8-9 mo old). Rats were anesthetized with pentobarbital and the stomach tissue was transcardially perfused with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution and dissected. Animal protocols were approved by the Animal Research Committee of Osaka Prefecture University.

Experimental induction of gastric ulcers

Gastric ulcers were induced with acidified ethanol solution using an established method[28] with minor modifications. Rats weighing 190-210 g were fasted for 24 h but allowed free access to drinking water; 1 mL acidified ethanol solution (80% ethanol in 0.15 mol/L HCl) was orally administrated to each animal using a disposable feeding needle (Fuchigami Ltd., Muko, Japan) on day 0. Stomach tissue samples were processed for histological examination by staining with hematoxylin and

eosin (H-E) on days 1, 3, 7, 10, and 14. A 100% survival rate was observed among rats that developed gastric ulcers, and epithelial regeneration was observed by day 3 in stained specimens. Ulcers and regenerating regions were randomly distributed on the mucosal surface of the stomach, which were easily identifiable by naked eye until at least day 7 as small, pale, or petechial/ecchymotic hemorrhagic puncta. Regenerating regions could not be clearly distinguished after day 10 by naked eye; thus, day 7 was selected for histochemical and RT-PCR analyses of regenerating gastric epithelium.

Experimental induction of gastric dysplasia

Dysplasia of gastric glandular epithelia was induced by a previously described method[29] with minor modifications. Briefly, rats weighing 190-210 g were fasted for 24 h but allowed free access to drinking water. A single dose (250 mg/kg) of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Kanto Chemical Co., Tokyo, Japan) prepared at 50 mg/mL in 75% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, United States) was orally administrated to rats using a disposable feeding needle; 6-7 mo later, rats were sacrificed and their stomachs dissected and fixed. Concave/convex regions on the mucosal surface were cut with a razor blade and processed for histochemistry.

Gastric cancer cell lines and cell culture

The human gastric carcinoma cell lines AZ-521, Kato-III, MKN-7, and SH-10-TC were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). AZ-521 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Cell Culture Bioscience, Nichirei Biosciences Inc., Tokyo, Japan) and 100 IU/mL penicillin and 100 μg/mL streptomycin (pen/strep; Sigma-Aldrich). Kato-III, MKN-7, and SH-10-TC cells were cultured in Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich) containing 10% FBS and pen/strep. Cells were maintained in a humidified 5% CO_2/95% air incubator at 37 ℃ and used in RT-PCR experiments.

Antibodies

Goat polyclonal antibodies against the extracellular domains of mouse EphB2 (AF467), EphB3 (AF432), EphB4 (AF446), and ephrin-B1 (AF473) were obtained from R&D Systems, Inc. (Minneapolis, MN, United States). A rabbit polyclonal antibody against the ephrin-B carboxy terminus (anti-ephrin-B1/B2/B3, C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). This antibody was used to detect the combined expression of ephrin-B ligands. The rabbit anti-human Ki67 monoclonal antibody (SP6) was from NeoMarkers, Inc. (Fremont, CA, United States). Biotinylated rabbit anti-goat and goat anti-rabbit IgG were from Vector Laboratories, Inc. (Burlingame, CA, United States), and Alexa Fluor 488-conjugated donkey anti-goat IgG was from Molecular Probes, Inc. (Eugene, OR, United States).

RT-PCR

Total RNA was isolated from normal gastric corpus mucosa and regenerating mucosa on day 7 after ulcer induction, as well as human gastric carcinoma cell lines, using Trizol reagent (Invitrogen Japan K.K., Tokyo, Japan). RT-PCR was performed as previously described[30]. Briefly, 1 μg total RNA was transcribed into first-strand cDNA by using M-MLV reverse transcriptase, RNase H$^-$ (Promega, Madison, WI, United States) and oligo(dT)$_{18}$ primer, according to the manufacturer's instructions. For the detection of endogenous EphB receptors and ephrin-B ligands, 1 μL of the reaction mix (from a total of 25 μL) was amplified over 36 cycles with cDNA as the template. The RT reaction was omitted for the negative control sample. Expression levels of amplified rat EphB1-B4 and B6, and ephrin-B1 and -B2 mRNA were determined from three independent experiments and normalized to the levels of β-actin mRNA as an internal control (amplified over 23 cycles). Expression levels of human GAPDH mRNA (amplified over 23 cycles) were used as an internal control for those of amplified human EphB1-B4, B6, and ephrin-B1 and -B2 mRNA in the samples of human gastric carcinoma cell lines. Primer sequences used to amplify rat EphBs and ephrin-Bs were as previously described[11], and forward and reverse primers for rat β-actin and human EphBs, ephrin-Bs and GAPDH were as follows: β-actin, 5'-GGC ATC CTG ACC CTG AAG TA-3' and 5'-TCT CAG CTG TGG TGG TGA AG-3'; EphB1, 5'-AAT GGC ATC ATC CTG GAC TA-3' and 5'-TCA ATC TCC TTG GCA AAC TC-3'; EphB2, 5'-CAA TGC GGA AGA GGT GGA TG-3' and 5'-GGA TCT CGA AGG TGT ACT GG-3'; EphB3, 5'-GTG AGT GGC TAC GAT GAG G-3' and 5'-GGA GAT GAG CGA CAT GCA G-3'; EphB4, 5'-GCA GTT CTC TGC CTC AGG A-3' and 5'-GCT CGA ACT GGC CCA TGA T-3'; EphB6, 5'-CTG AGA GCC GAG TGT TAG TGG-3' and 5'-AGC TCC CCT TGA GGA AGT GTC-3'; ephrin-B1, 5'-TCA ACC CCA AGT TCC TGA GTG-3' and 5'-GCG TAG CTT CAG TAG TAG GAC-3'; ephrin-B2, 5'-ACC CAC AGA TAG GAG ACA AA-3' and 5'-GGT TGA TCC AGC AGA ACT TG-3'; ephrin-B3, 5'-CCT AAC CAG AGG CAT GAA GG-3' and 5'-TCT CAT AGT GGG GGC AGA AG-3'; and GAPDH, 5'-GTC GGA GTC AAC GGA TTT GG-3' and 5'-GGA TGA TGT TCT GGA GAG CC-3'.

Immunolabeling and lectin fluorescence staining

The stomachs of normal rats, and those with experimentally induced ulcers or dysplasia were cut into 3- to 5-mm-thick pieces that were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h at 4 ℃. After washing with PBS, the pieces were immersed in 30% sucrose in PBS overnight, embedded in Optimal Cutting Temperature compound (Sakura Finetechnical

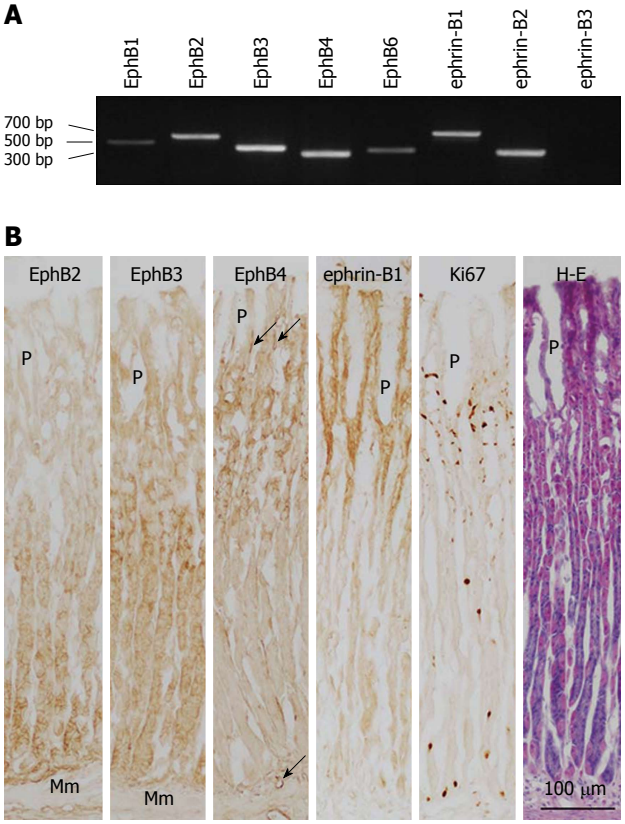

Figure 1 Expression of B-class Eph receptors and ephrin ligands in normal rat gastric corpus mucosa. A: EphB and ephrin-B mRNA expression was determined by reverse transcription-polymerase chain reaction; B: Expression of EphB2-B4, ephrin-B1, and Ki67 in the gastric corpus mucosa was evaluated by immunoperoxidase and hematoxylin and eosin co-staining of frozen sections. Mm: Muscularis mucosae; P: Gastric pit; arrow: Blood vessel.

Co., Ltd., Tokyo, Japan), and sectioned at a thickness of 6-7 μm on a cryostat. Sections were stained by the immunoperoxidase method[30]. Briefly, sections were immersed in 0.3% hydrogen peroxide for 30 min, preincubated with 3% normal rabbit or goat serum in PBS, then incubated with primary antibodies against EphB2, B3, or B4, ephrin-B1 or ephrin-B1/B2/B3 (all at 1 μg/mL) or Ki67 (1:200) overnight at 4 ℃. Avidin and biotin (Avidin/Biotin Blocking Kit; Vector Laboratories, Inc., Burlingame, CA, United States) were added to the blocking and primary antibody solutions, respectively, to block endogenous binding sites. Sections were incubated with biotinylated rabbit anti-goat or goat anti-rabbit IgG, followed by treatment with an avidin-biotin peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Inc.), and they were developed by immersion in 3,3'-diaminobenzidine substrate (KPL, Gaithersburg, MD, United States). The specificity of the staining was verified by incubation without primary or secondary antibodies. Some immunoperoxidase staining sections were counterstained with haematoxylin. A subset of sections was stained with H-E.

Fluorescein-conjugated *Griffonia simplicifolia* (GS)-Ⅱ lectin and rhodamine-conjugated *Ulex europaeus* agglutinin (UEA)-I lectin (both from Vector Laboratories, Inc.) were used to identify mucous neck and pit cells (*i.e.*, surface mucous cells), respectively[11]. Fluorescence co-labeling of lectins and EphB2 was carried out as previously described[9]. Cryostat sections were preincubated in a humid chamber with 1% bovine serum albumin in PBS, followed by incubation with the primary antibody against EphB2 (2 μg/mL) for 1 h at 32 ℃. After washing with PBS, sections were incubated with a mixture of Alexa Fluor 488-conjugated donkey anti-goat IgG and UEA-I (1 μg/mL) for 30 min at 32 ℃, and following PBS washes, mounted with Permafluor (Immunotech, Marseille, France). A subset of sections was double-labeled with GS-Ⅱ (1 μg/mL) and UEA-I for 30 min at 32 ℃. Sections were imaged with a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed with StatView software (SAS Institute Inc., Cary, NC, United States). Histograms show mean ± SD. An unpaired *t*-test was used to compare means and a P value < 0.05 was considered statistically significant.

RESULTS

Expression and localization of EphB and ephrin-B in normal gastric mucosa

Tissue samples of gastric corpus mucosa in normal adult rats were screened by RT-PCR to identify EphB receptors and ephrin-B ligands expressed in the adult stomach. With the exception of ephrin-B3, transcripts for all mammalian EphB and ephrin-B molecules were detected (Figure 1A), with the most prominent expression observed for EphB2-B4, and ephrin-B1 and -B2.

Immunoperoxidase staining was performed to determine the localization of the most abundant receptors and ligands. The isthmus, where proliferating cells are localized, was identified by Ki67 immunoreactivity. EphB receptors and ephrin B ligands were expressed in the plasma membrane of cells. EphB2 was expressed in gland cells, strongly in the base region where chief cells are located and weakly in the lower portion of the neck (Figure 1B). EphB3 expression was stronger in the neck of gastric glands and weaker in the isthmus and base region. EphB4 was expressed in the isthmus and neck of gastric glands and in blood vessels of the lamina propria mucosae. In contrast, ephrin-B1 immunoreactivity was observed in pit cells lining the lumen, gastric pits, and in cells located in the isthmus (Figure 1B). A similar labeling pattern was seen using the ephrin-B1/B2/B3 antibody, which recognizes all three ephrin-B ligands (data not shown). Thus, in gastric units, pit cells and proliferating isthmus cells express ephrin-B. In summary, cells located in the epithelium overlying the lumen, gastric pits, and isthmus expressed ephrin-B ligands, whereas cells in the neck and based of gastric glands and isthmus expressed EphB receptors; thus, receptors and ligands are differentially expressed within gastric units but are coexpressed in the

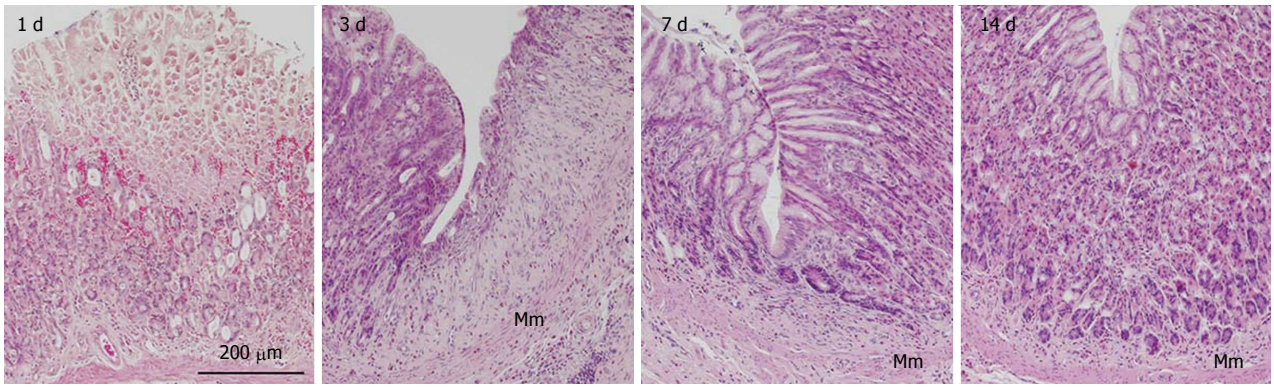

Figure 2 Representative images of rat gastric corpus mucosa showing typical regeneration 1, 3, 7, and 14 d after induction of gastric ulcers by oral administration of acidified ethanol solution (80% ethanol in 0.15 mol/L HCl). Tissue sections were stained with hematoxylin and eosin. Mm: Muscularis mucosae.

isthmus, where undifferentiated gastric stem cells and transit-amplifying cells reside.

Expression and localization of EphB and ephrin-B in regenerating gastric mucosa

Expression profiles of EphB and ephrin-B were examined by RT-PCR and immunolabeling in an experimental ulcer model to determine whether complementary receptor/ligand expression patterns are maintained in an actively regenerating gastric epithelium. Gastric corpus mucosa underwent a typical regeneration process 3-14 d after induction of gastric ulcers, as observed in H-E-stained sections. On day 3, epithelial cells covered the luminal surface of the gastric mucosa (Figure 2); on day 7, gastric units composed of incomplete and short gastric glands, with gland cells strongly stained with eosin, were formed in the regenerating mucosa with thick stromal tissues. On day 14, the regeneration of gastric mucosa was almost complete and the regenerated tissue could not be histologically distinguished from normal tissue except for the presence of a relatively abundant stroma in the former. Based on histological assessment of the regeneration process, the gastric mucosa on day 7 was deemed suitable for analysis of EphB and ephrin-B expression because at this time point there were clear signs of active epithelial regeneration.

The mRNA expression of all EphB receptors and ephrin-B ligands was assessed with the exception of ephrin-B3, for which no expression was observed in either the control or ulcerated gastric corpus mucosa on day 7 by RT-PCR. The expression levels of all molecules were similar to those of the control at this stage of regeneration except for EphB2, which was upregulated by 2.1-fold ($P = 0.016$; Figure 3).

Immunoperoxidase staining, and immuno- and lectin fluorescence labeling of serial sections were used to determine the localization and changes in expression of the most abundant EphB receptors and ephrin-B ligands in the regenerating gastric corpus mucosa on day 7. Ki67-positive proliferating cells were abundant in the regenerating gastric mucosa, *i.e.*, regenerating gastric units from the base of the gastric pits to the bottom of the gastric glands, as well as in stromal cells (Figures 4 and 5). In regions adjacent to the regenerating gastric mucosa where gastric units elongated to a length similar to the control, Ki67-positive cells were mainly restricted to the isthmus. UAE- I -positive cells formed gastric pits and covered the luminal surface, whereas by lectin fluorescence labeling, GS- Ⅱ -positive cells were detected throughout the gastric glands in regenerating gastric units (Figure 5). Thus, at this time point, Ki67-positive mucous neck cells had emerged to form the entire length of regenerating gastric glands, which were still extremely thin and lacked a base and part of the neck. EphB2 and B3 were localized in the plasma membrane of gland cells. EphB2 was not detected in control, but was upregulated in gastric units of regenerating areas on day 7 (Figure 4) and detected not only in gastric glands but also in the isthmus and gastric pits occupied by Ki67-positive cells (Figure 5). EphB3 was also upregulated in gastric units of regenerating areas in the isthmus and gastric glands, in contrast to its localization in the control mucosa (Figure 4). The EphB4 expression pattern was similar in regenerating and control mucosa, and was mainly seen in the isthmus and gastric glands of regenerating gastric unit as well as in blood vessels. Ephrin-B1 immunoreactivity was similar in regenerating and control mucosa, and was seen mainly in pit cells (Figure 4). In the regenerated gastric mucosa on day 14, localization of the EphB receptors and ephrin-B1 were the same as that in control rats (data not shown). Thus, the ephrin-B1-expressing region temporarily overlaps with the upper part of the region that highly expresses EphB2 in regenerating gastric units.

EphB and ephrin-B localization in the gastric epithelial dysplasia

The expression of EphB and ephrin-B in aberrantly proliferating gastric epithelium was examined by immunoperoxidase staining of experimentally induced gastric dysplasia. A single sublethal dose of MNNG induced dysplastic lesions in the rat gastric mucosa several months later, where hemorrhage was histologically identified and numerous Ki67-positive cells were present,

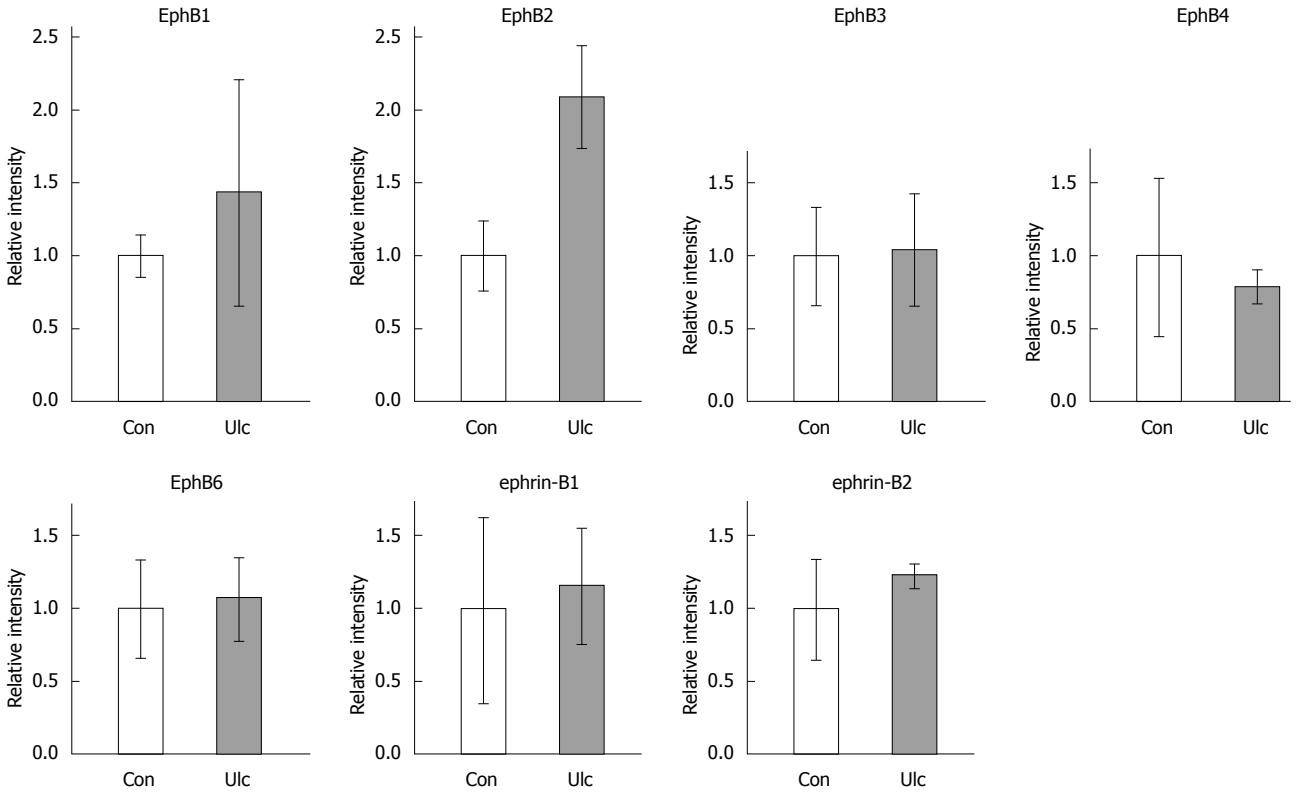

Figure 3 Densitometric quantification of EphB1-B4 and B6, and ephrin-B1 and -B2 mRNA expression levels in the gastric corpus mucosa of control (Con) and ulcerated regenerating stomach on day 7 after induction of gastric ulcers (Ulc), as determined by reverse transcription-polymerase chain reaction. EphB2 expression in the regenerating gastric mucosa was higher than in the control (P = 0.016; unpaired t-test).

comprising multiple layers in dysplastic epithelia (Figure 6). EphB receptors (EphB2-B4) and ephrin-B1 were highly expressed in the plasma membrane of gastric cells in Ki67-positive and -negative regions (Figure 6); in the former, ephrin-B1 was also detected in the cytoplasm of these cells. EphB4 was also highly expressed in blood vessels. These results demonstrate an overlap of regions expressing high levels of EphB and ephrin-B in gastric dysplasia, suggesting that dysplastic cells coexpress both receptors and ligand. Expression patterns of EphB receptors, ephrin-B1 ligand, and Ki67-positive cells in normal, ulcerated regenerating, and dysplastic gastric epithelia are summarized in Figure 7.

EphB and ephrin-B expression in gastric cancer cell lines

EphB and ephrin-B mRNA expression was examined by RT-PCR in four human gastric carcinoma cell lines. EphB1 and ephrin-B2 were prominently expressed, and EphB2 and B3, and ephrin-B1 were weakly expressed, in AZ-521 cells. In KATO-III cells, EphB2, and ephrin-B1 and -B2 transcript levels were high, while EphB1, B3, and B4 mRNA was detected at low levels. MKN-7 cells highly expressed EphB2 and B3 and ephrin-B2, while weak expression of EphB1 and B6 was also seen. High levels of EphB1, B2, B3, and B6 and ephrin-B2, and relatively low levels of EphB4 and ephrin-B1 were observed in SH-10-TC cells (Figure 8). These results suggest that unlike in normal gastric epithelium, ephrin-B3 is not expressed in gastric carcinomas; and gastric carcinoma cells coexpress more than one EphB receptor and ephrin-B ligand.

DISCUSSION

Eph receptor tyrosine kinases and their ephrin ligands function in cell-cell communication, with widespread roles in the development and physiology of various tissues and organs[1,3]. The present study examined whether EphB and ephrin-B expression profiles can serve as an indicator of dysplastic/oncogenic transformation in gastric mucosa. This is the first comprehensive analysis of the expression profiles of B-class Eph receptors and ephrin ligands in normal, ulcerated regenerating, and dysplastic gastric mucosa in a rodent model and human gastric carcinoma cell lines. The ephrin-B- and EphB-expressing regions - in the upper (luminal surface and gastric pits) and lower (gastric gland) compartments, respectively - are divided along the pit-gland axis in normal gastric units, consistent with our previous findings in the mouse[9-11]. We also found that the time-dependent upregulation of EphB2 and B3 expression, as evidenced by immunohistochemical signal intensity, were similar in regenerating and normal, adjacent gastric units. Notably, the region highly expressing EphB2 extended up to the gastric pits and/or the luminal surface where UEA-I-/Ki67-positive pit cells reside. This suggests that proliferating cells in the isthmus and gastric pits

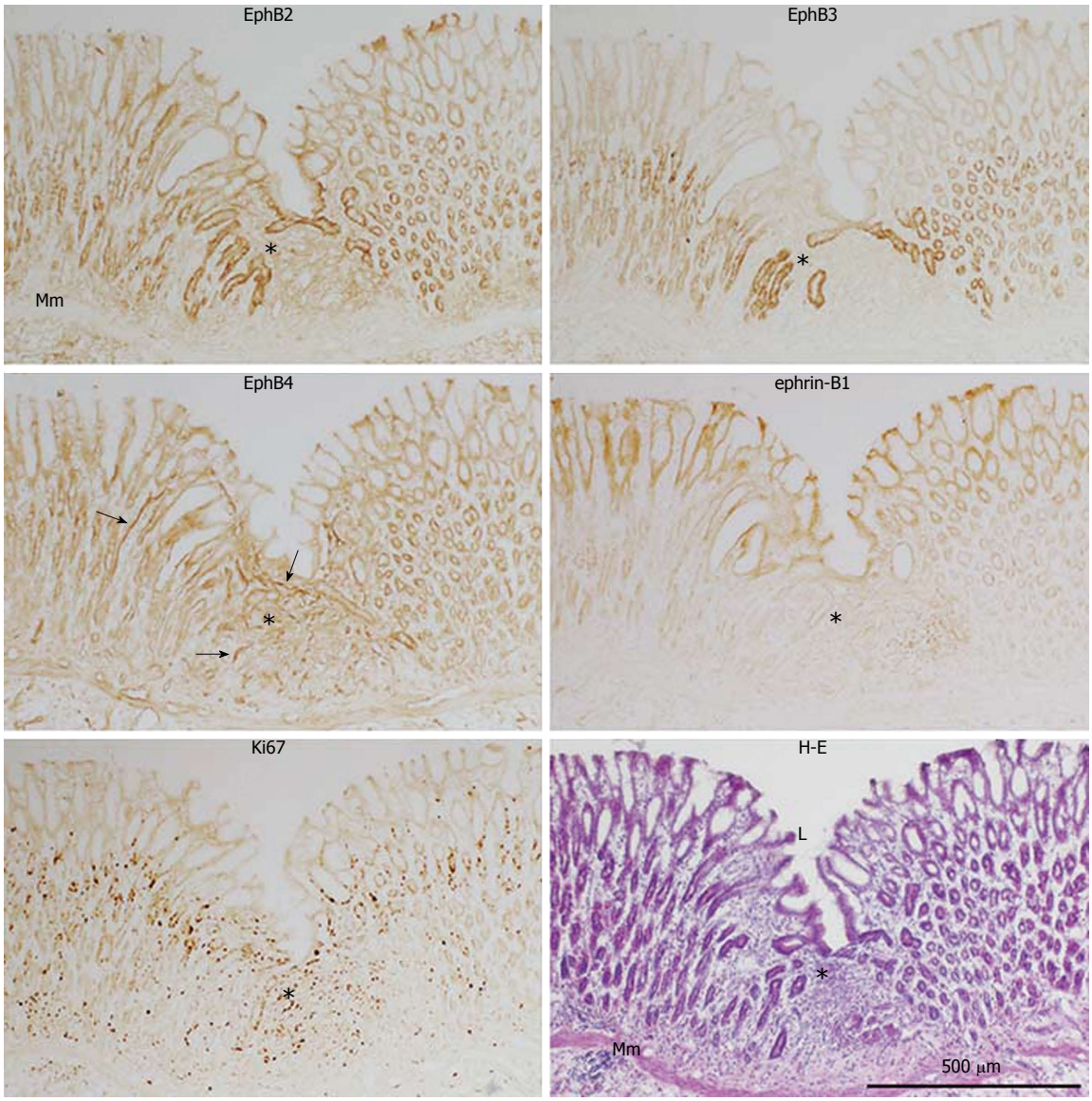

Figure 4 Immunoperoxidase staining of EphB2, B3, and B4, ephrin-B1, and Ki67, with hematoxylin and eosin staining, of frozen sections of the regenerating gastric corpus mucosa 7 d after experimental induction of gastric ulcers (see Figure 2 legend). Mm: Muscularis mucosae; L: Gastric lumen; Arrow: Blood vessel; Asterisk: Regenerating region.

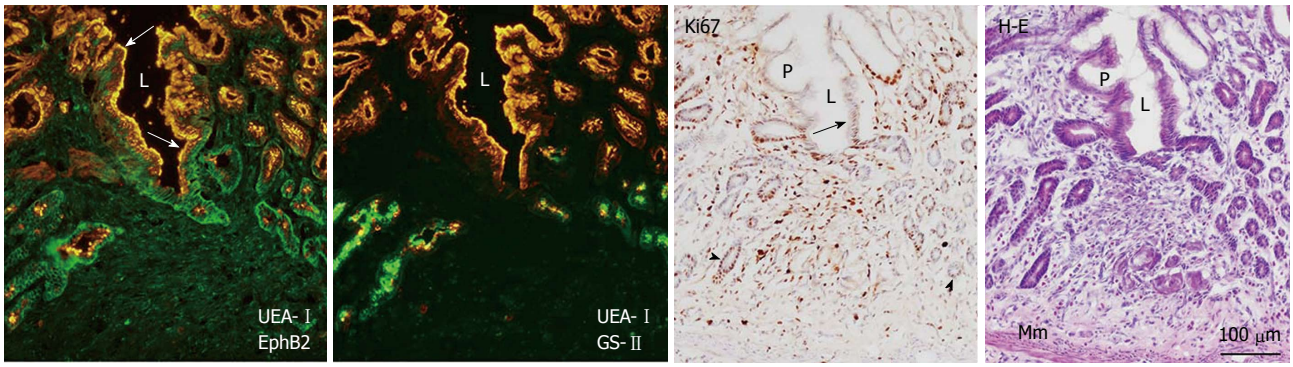

Figure 5 Double immunofluorescence labeling of EphB2 (green) and the pit cell marker *Ulex europaeus* agglutinin (UEA)- I (orange), and the neck cell marker *Griffonia simplicifolia*- II (green) and UEA- I , with Ki67 immunoperoxidase and hematoxylin and eosin co-staining in frozen sections of the regenerating gastric corpus mucosa 7 d after induction of gastric ulcers (see Figure 2 legend). L: Gastric lumen; Mm: Muscularis mucosae; P: Gastric pit; Black arrow: Ki67-positive pit cells; Arrowhead: Ki67-positive gland cells; White arrow: UEA- I -positive pit cells.

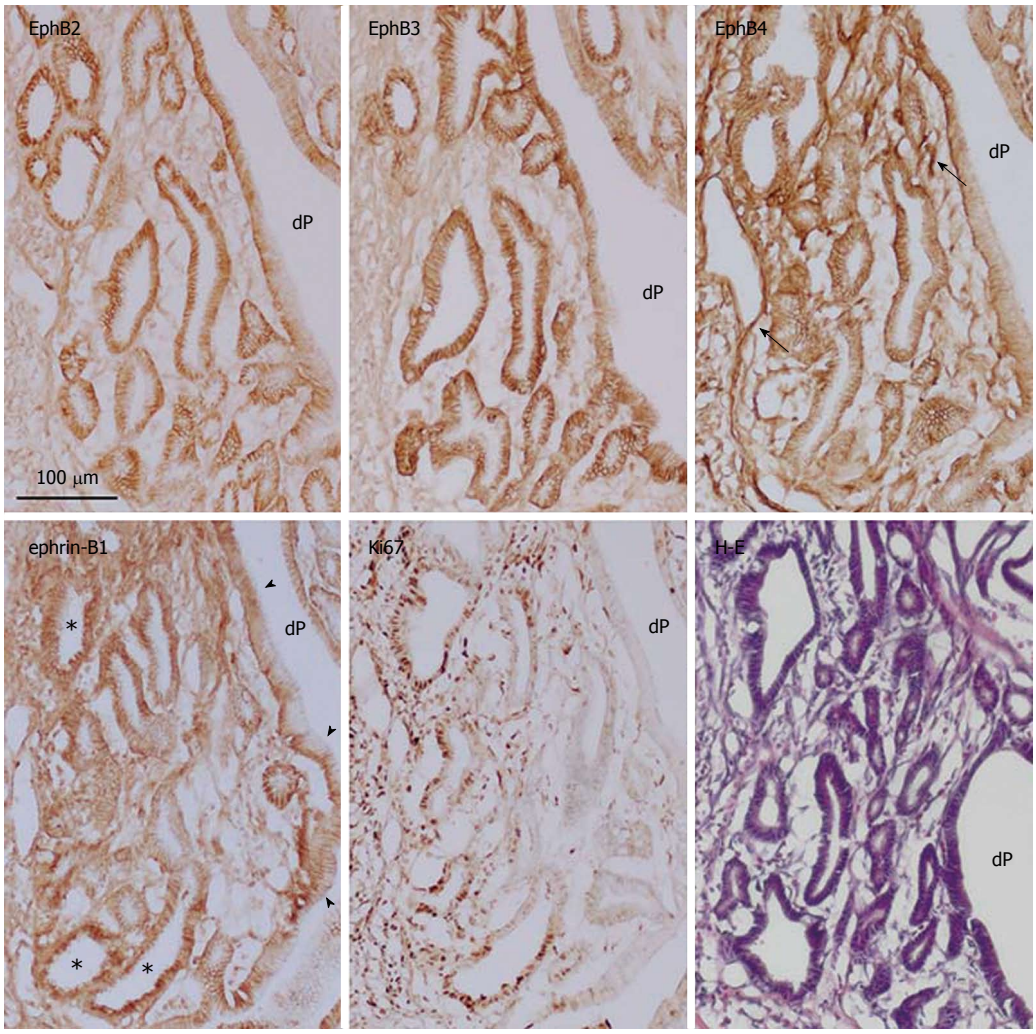

Figure 6 Immunoperoxidase staining of EphB2-B4, ephrin-B1, and Ki67, and hematoxylin and eosin staining of frozen sections of gastric dysplasia in rat induced by oral administration of N-methyl-N'-nitro-N-nitrosoguanidine. dP: Deep and wide aberrant gastric pit; Arrow: Blood vessel; Arrowhead: Membrane localization of ephrin-B1; Asterisk: Cytoplasmic localization of ephrin-B1.

temporarily upregulate EphB2 expression during the normal regeneration process. Because there was no alteration in ephrin-B1 signal intensity in these cells, EphB2 and ephrin-B1 are likely coexpressed at constant, high levels in gastric units at this time. Moreover, EphB receptors (EphB2-B4) and ephrin-B1 were upregulated and ubiquitously distributed throughout gastric cells in the dysplastic mucosa, where the Ki67-positive cell region extended down to the bottom of epithelial invaginations and dysplastic cells did not form clear gastric units. This indicates that EphB receptors and ephrin-B1 are coexpressed at high levels in aberrantly proliferating cells in the gastric dysplasia. Based on these findings, it was hypothesized that the emergence of proliferating cells that upregulate expression of both EphB receptors and ephrin-B ligands is an indication of dysplastic/oncogenic transformation. The observed patterns of receptor and ligand mRNA expression in four human gastric carcinoma cell lines provided evidence in support of this hypothesis. Thus, the coexpression of EphB receptors and ephrin-B ligands is also a likely feature of gastric carcinoma. We previously detected the coexpression of certain Eph receptors and ephrin ligands in human tumor samples[31], which suggested that their coexpression in tumors may not be atypical. A comprehensive analysis of receptor-ligand coexpression profiles in human gastric carcinoma samples is required to determine whether this is indeed the case.

Canonical Eph forward signaling has tumor suppressor function, based on studies in which forced Eph activation by soluble and dimerized ephrins inhibited proliferation, survival, migration, and invasion of various types of tumor cell in culture and tumor progression in several mouse models[5]. Certain Eph receptors and ephrin ligands are expressed in vascular endothelial cells in human tumor samples[3,31] and are presumed to promote tumor angiogenesis and thus its progression[5], while dysregulation of Eph/ephrin signaling affects tumor progression and metastasis *in vitro* and *in vivo*[4,5,19]. In the present study, the coexpression of more than one receptor and/or ligand was observed in dysplastic gastric cells and gastric carcinoma cell lines. This can lead to

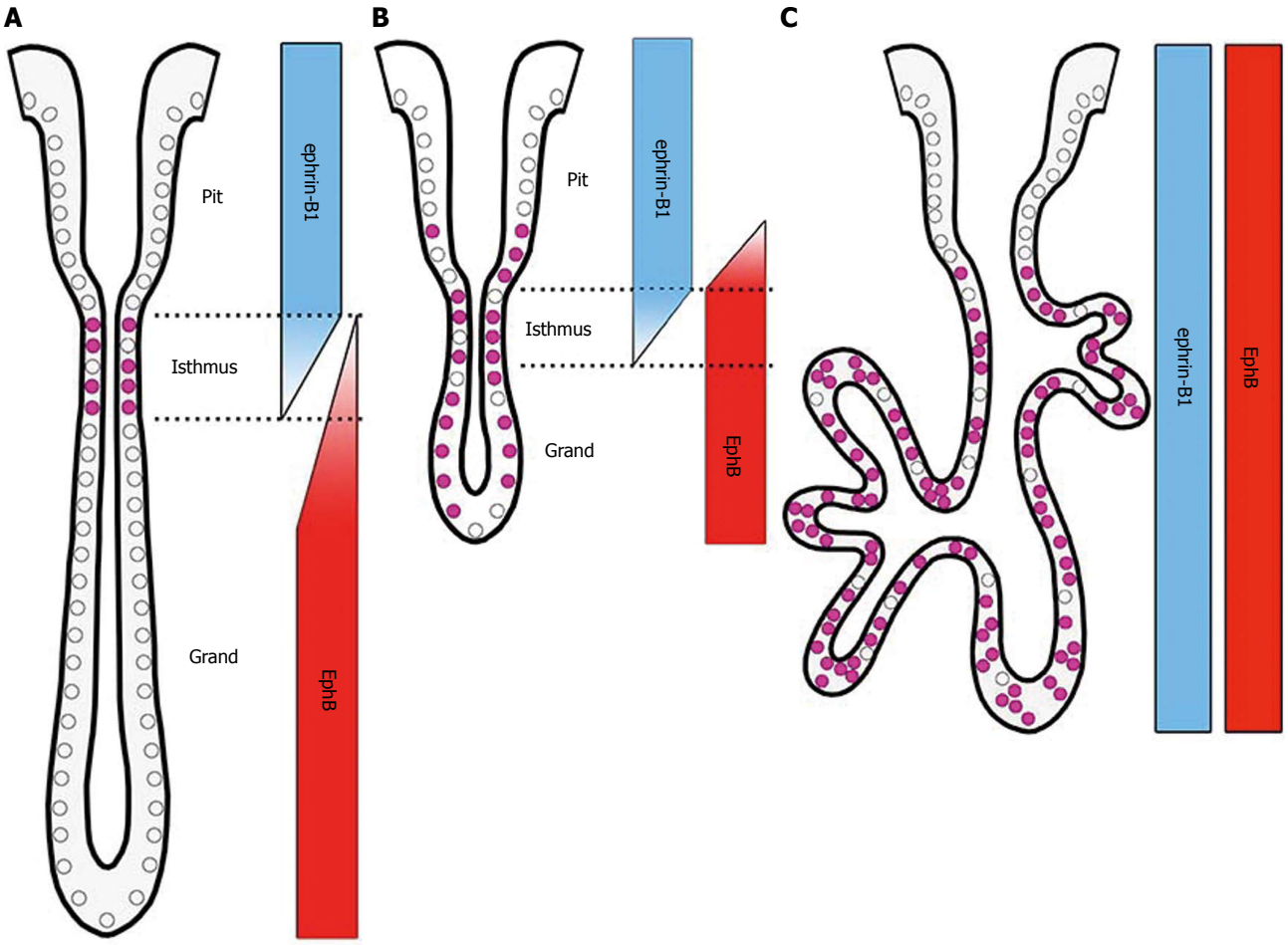

Figure 7 Schematic representation of EphB receptor, ephrin-B1 ligand, and Ki67 expression in normal (A), ulcerated regenerating (B), and dysplastic gastric epithelia (C).

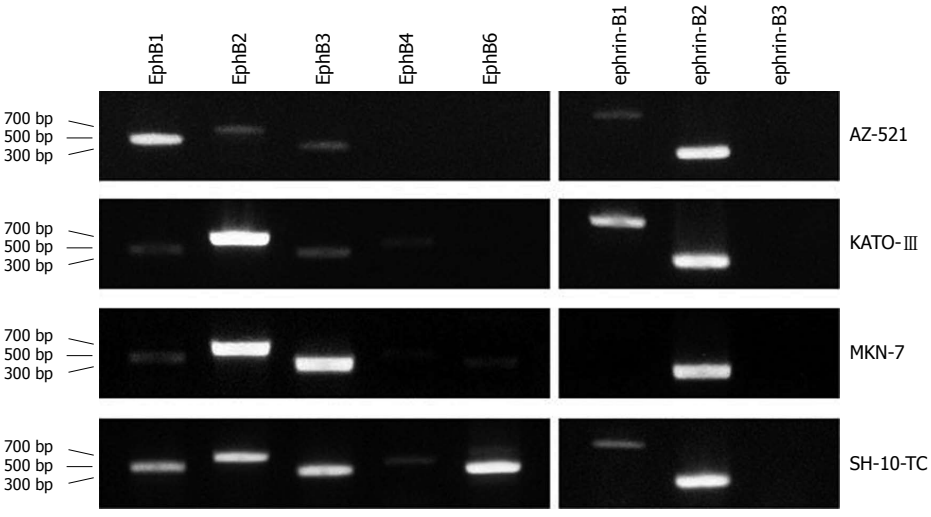

Figure 8 EphB and ephrin-B mRNA expression in human gastric carcinoma cell lines. Transcript levels in AZ-521, KATO-Ⅲ, MKN-7, and SH-10-TC cells were determined by reverse transcription-polymerase chain reaction. All cell lines coexpressed more than one EphB receptor and/or ephrin-B ligand at a high level.

ineffectual lateral interactions in tumor cells by mutual inhibition in intercellular interactions[2,5,32]. It is well known that EphB receptors may be overexpressed during early stages of tumor progression and subsequently downregulated at the adenoma-carcinoma transition in colorectal tumors[26]. In mouse colorectal tumor models, the expansion and invasiveness of the initial tumor cells expressing EphB receptors at high levels were inhibited

by contact with ephrin-B-expressing normal cells enclosing the tumor compartment: the forward signaling caused cell-cell repulsion between tumorigenic and normal cells, as well as adhesion between tumor cells[26,27].

Less attention has been given to the EphB receptors in gastric tumors. The overexpression or reduced and/or loss of EphB1 and B2 expression has been reported in human gastric tumor samples relative to adjacent normal tissue, which has been correlated with gastric cancer progression, metastasis, and poor prognosis[20,24]. Thus, as in the case of colorectal carcinoma, early upregulation of EphB receptor expression followed by a downregulation at the adenoma-carcinoma transition likely occurs in gastrointestinal tumors. However, EphB-expressing cells located in the basal region of dysplastic gastric mucosae were not surrounded by cells expressing normal levels of ephrin-B, unlike the localization of receptors and ligands in the early stage of colorectal tumors. Moreover, EphB and ephrin-B coexpression was observed in all gastric carcinoma cell lines examined in the present study, implying that EphB receptors may not be acting as tumor suppressors through lateral inhibition. Nonetheless, the coexpression of EphB/ephrin-B may be a useful marker for gastric tumor progression; further investigation will be required to determine whether, for instance, tyrosine phosphorylation status in these proteins is correlated with expression profiles and tumor progression.

Xenograft studies have shown that ephrin-B1 signaling can promote gastric tumor cell invasion[33,34], and is therefore likely implicated in tumor progression. The present investigation detected cells strongly coexpressing EphB receptors and ephrin-B1 in dysplastic Ki67-positive and adjacent Ki67-negative regions; ephrin-B1 was also frequently localized in the cytoplasm of proliferating cells. Eph-ephrin complexes and surrounding plasma membrane are internalized *via* endocytosis in cells expressing either the receptor or ligand. This is the terminal event in the Eph-ephrin interaction, after which Eph forward and/or ephrin reverse signaling drives cell-cell repulsion[2,3], thereby blocking intercellular communication *via* the loss of cell-cell adhesion. Thus, the cytoplasmic localization of ephirn-B1 in dysplastic proliferating cells, but not in normal cells or regenerating gastric cells, could mark the progression of gastric tumors.

In conclusion, a constant and high level of EphB and ephrin-B coexpression was identified as a feature common to experimentally induced gastric dysplasia and human gastric carcinoma cell lines, as compared to normal gastric and ulcerated regenerating epithelia. Based on these findings, it is proposed that the stable and robust upregulation of coexpressed EphB and ephrin-B is a marker of dysplastic/oncogenic transformation. Eph forward signaling in tumor cells likely has a suppressive role during tumor progression, with Eph and ephrin coexpressed on the same cell engages in non-productive interactions *via* lateral inhibition and thereby silencing downstream signaling. These results can be useful for the early and accurate diagnosis of gastric tumors for the timely initiation of therapeutic interventions.

COMMENTS

Background

Up- or down-regulation in expression and mutations in genes of Eph receptors or ephrin ligands have been reported in human gastric tumors, and EphA overexpression in tumors is correlated with cancer progression, metastasis, and/or poor prognosis. Less attention has been given to EphB in gastric tumors while in colorectal tumors the expression of EphB receptors is high during early stages of tumor progression and downregulated at the adenoma-carcinoma transition.

Research frontiers

Canonical Eph forward signaling has tumor suppressor function, based on studies in which forced Eph activation inhibited proliferation, survival, migration, and invasion of various types of tumor cell in culture and tumor progression in several mouse models. Certain Eph receptors and ephrin ligands are expressed in vascular endothelial cells in human tumor samples and are presumed to promote tumor angiogenesis and thus its progression, while dysregulation of Eph/ephrin signaling affects tumor progression and metastasis *in vitro* and *in vivo*.

Innovations and breakthroughs

To determine whether EphB and ephrin-B expression profiles can be used as markers for dysplastic/oncogenic transformation in gastric mucosa, the protein expression and localization of EphB and ephrin-B in normal, ulcerated regenerating, and dysplastic gastric mucosa were examined in a rat experimental model by immunolabeling, and mRNA expression was assessed in four human gastric carcinoma cell lines by reverse transcription-polymerase chain reaction (RT-PCR).

Applications

Robust and stable coexpression of EphB and ephrin-B was a feature common to experimentally induced gastric dysplasia and human gastric carcinoma cell lines as compared to normal gastric and ulcerated regenerating epithelia. Thus, EphB/ephrin-B could be a useful marker combination for dysplastic/oncogenic transformation in gastric cancer.

Terminology

Eph receptors and ephrin ligands: The Eph receptor tyrosine kinases have 14 members, EphA (A1-A8 and A10) and EphB (B1-B4 and B6), in mammals. EphAs and EphBs promiscuously bind ephrin-A (A1-A5) and ephrin-B (B1-B3) ligands, respectively. Ephrin-As are glycosylphosphatidylinositol-anchored membrane proteins while ephrin-Bs are transmembrane proteins. The Eph/ephrin interaction results in bidirectional signal propagation in both receptor- and ligand-expressing cells. Eph forward signaling depends mainly on autophosphorylation and association of the receptor with various effector proteins, while ephrin reverse signaling in part on tyrosine phosphorylation of the cytoplasmic region of ephrin-Bs and associated proteins.

Peer review

This paper examined the expression profiles of EphB and ephrin-B in normal, ulcerated regenerating, and dysplastic gastric mucosa in a rat experimental model as well as human gastric carcinoma cell lines by immunolabeling and/or RT-PCR. The authors found a constant/high level of EphB and ephrin-B coexpression identified as a feature common to experimentally induced gastric dysplasia and human gastric carcinoma cell lines, as compared to normal gastric and ulcerated regenerating epithelia. These results may be useful for the early and accurate diagnosis of gastric tumors.

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ORIGINAL ARTICLE

Basic Study

Butein effects in colitis and interleukin-6/signal transducer and activator of transcription 3 expression

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Abstract

AIM: To evaluate the effects of butein on inflammatory cytokines, matrix metalloproteinase-9 (MMP-9), and colitis in interleukin (IL)-10$^{-/-}$ mice.

METHODS: To synchronize colitis, 8- to 10-wk-old IL-10$^{-/-}$ mice were fed pellet-chow containing piroxicam for 2 wk. Subsequently, phosphate-buffered saline or butein (1 mg/kg per day, ip) was injected for 4 wk. Histologic scores, inflammatory cytokines, MMP-9 and phosphorylated signal transducer and activator of transcription 3 (pSTAT3) expressions were analyzed in IL-10$^{-/-}$ mice and in Colo 205 cells.

RESULTS: Butein reduced the colonic inflammatory score by > 50%. Expression levels of IL-6, IL-1β, interferon (IFN)-γ and MMP-9 were decreased in the colons of mice exposed to butein, whereas other inflammatory cytokines (IL-17A, IL-21 and IL-22) were unchanged. Immunohistochemical staining for pSTAT3 and MMP-9 was significantly decreased in the butein-treated groups compared with the controls. Butein inhibited IL-6-induced activation of STAT3 in Colo 205 cells.

CONCLUSION: Butein ameliorated colitis in IL-10$^{-/-}$ mice by regulating IL-6/STAT3 and MMP-9 activation.

Key words: Butein; Interleukin-6/signal transducer and activator of transcription 3; Colitis; Inflammatory bowel disease; Matrix metalloproteinase-9

Core tip: This study examined if butein, a naturally derived substance, has therapeutic effects in an animal model of inflammatory bowel disease, interleukin (IL)-10$^{-/-}$ mice. The results show that butein suppressed bowel inflammation and interfered with the IL-6/signal transducer and activator of transcription 3 and matrix metalloproteinase-9 pathways, suggesting that butein

should be used to treat bowel inflammation-induced colon cancer. Although there have been several *in vitro* studies on tumor cells, there have been no *in vivo* studies examining the effect of butein on colitis in mice.

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INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, are chronic diseases that are occasionally complicated by bowel perforations, strictures and fistulas[1,2]. The overall risk of colorectal cancer among patients with ulcerative colitis is about ten times higher than that of the general population[3,4]. In Asia, including South Korea, the occurrence of colon cancer and IBD has recently increased due to environmental factors such as the influence of Westernized lifestyles. This incidence pattern is expected to continue[2]. Immunosuppressive agents and 5-amino-salicylic acid have classically been used as treatments for IBD. Inhibition of tumor necrosis factor (TNF), an inflammatory cytokine, has been considered as a therapeutic target. Many studies have focused on diverse biologic agents to treat IBD, but a completely efficient treatment agent has not yet been discovered. Research continues for the development of new alternative drugs and in clinical trials[5,6].

IBD results from immune modulation abnormalities; helper T cells, in particular, play a critical role in the development of disease. CD is largely associated with abnormal activation of Th1-related cytokines [interleukin (IL)-1β, interferon (IFN)-γ, TNF-α, IL-6 and IL-22]; in addition, the importance of Th17-related cytokines in the emergence of CD has recently been highlighted[7]. IL-10$^{-/-}$ mice are known to be an appropriate animal model for CD due to the similarity of their condition to CD morbidity. In these mice, the manifestation of bowel inflammation is mediated by Th1 cytokines (IL-1β, IFN-γ, TNF-α and IL-6), and the ulcerative lesions are found in the proximal portion of the colon. In IL-10$^{-/-}$ mice, chronic bowel inflammation can occur without any other stimuli or specific pathogens. Making use of the fact that bowel inflammation occurs after a certain period of time, it is possible to promote inflammation and inflammation-induced tumors with piroxicam, a non-steroidal anti-inflammatory drug. Therefore, the IL-10$^{-/-}$ mouse can be a useful animal model for the occurrence of bowel inflammation and inflammation-induced tumors[8,9].

The therapeutic effects of natural substances on inflammation and tumors are being widely studied[10,11]. It has been reported that a number of chemical substances obtained from edible plants with botanical and antioxidant characteristics interfere with tumorigenesis through suppression of the inflammatory reaction[12]. However, there has been no agreement in results between actual clinical applications and a large-scale prospective study[13]. In addition, numerous mechanisms have been suggested for the anti-inflammatory and anti-tumor effects of such natural substances. Cell signal transmission systems have been actively studied, but it has been difficult to elucidate the mechanism of the therapeutic effects due to the structural diversity[13]. *Toxicodendron vernicifluum*, a deciduous tree from the Anacardiaceae family, both grows natively in various places and is cultivated in South Korea. Urushiol, the major constituent, is primarily responsible for the toxicity. Other constituents, such as butein (3,4,2',4'-tetrahydroxychalcone), have been found in *in vitro* studies to have antioxidant and anti-inflammatory effects and to suppress tumor cell proliferation and angiogenesis[14-18]. One *in vitro* study demonstrated that butein inhibits the activation of nuclear factor kappa B (NF-κB) through inhibition of TNF-α, IL-6 and IL-8 in human mast cells[19].

Matrix metalloproteinase (MMP), an enzyme that degrades zinc-dependent gelatin matrices, serves an important role in inflammatory cell infiltration, cytokine activation and tissue injury, reformation and recovery. MMP-9 is specifically known to be closely associated with rheumatoid arthritis, atherosclerosis, colon cancer and IBD. The suppressive effects that butein has on MMP-9 activation have been reported in an *in vitro* study using prostate cancer cells[20,21]. However, to our knowledge, no study has yet been conducted that examines the therapeutic effects of butein on bowel inflammation and colon cancer. This study, therefore, aims to evaluate the interfering effects of butein on inflammatory cytokines and MMP-9 in IL-10$^{-/-}$ mice and ultimately to examine the therapeutic effects of butein.

MATERIALS AND METHODS

Experimental animals

IL-10$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME, United States). All mice were housed and bred in the animal care facility of Korea University Guro Hospital.

Reagents

Butein and piroxicam were purchased from Sigma-Aldrich Inc. (St. Louis, MO, United States). Butein was dissolved in dimethylsulfoxide and was diluted in phosphate-buffered saline (PBS) before use. Antibodies against phosphorylated signal transducer and activator of transcription 3 (pSTAT3)/STAT3 and β-actin were purchase from Cell Signaling Co. (Beverly, MA, United States), anti-MMP-9 was purchased from Abcam Inc. (Cambridge, United Kingdom), anti-BrdU was purchased from Accurate Chemical (Westbury, NY, United States),

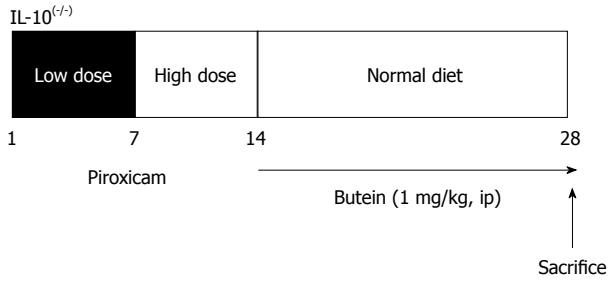

Figure 1 Experimental protocol for the interleukin-10$^{-/-}$-piroxicam colitis model. IL: Interleukin; ip: Intraperitoneal injection.

GAPDH and donkey anti-rabbit antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, United States), and anti-rabbit and mouse-HRP-labeled polymers were purchased from Dako of Agilent Technologies (Glostrup, Denmark).

Colitis induction

To synchronize and accelerate colitis, 8-wk-old IL-10$^{-/-}$ mice were treated with piroxicam as previously described[22,23]. In brief, a lower dose of piroxicam (60 mg/250 g chow) was administrated for 7 d followed by a higher dose of piroxicam (80 mg/250 g chow) for 7 d. Mice were then placed on normal chow for the remainder of the experimental period. On days 15-28, 1 mg/kg of butein or PBS was administered daily to mice, and mice were sacrificed on the 28th day (Figure 1). This experiment was performed in accordance with the guidelines of the Korea University Animal Ethics Committee.

Colitis assessment

Entire colons were dissected longitudinally and made into Swiss rolls. The tissues were fixed in 10% formalin for 14-16 h, embedded in paraffin and 4-μm sections were cut and stained with hematoxylin and eosin. The degree of colitis was assessed using the inflammation scoring system as described previously with slight modifications[24] (Table 1).

Cell lines and cultures

The human colon cancer cell line, Colo 205, was purchased from the American Type Culture Collection (Manassas, VA, United States). Cells were cultured in RPMI media containing 1% penicillin-streptomycin (Sigma) and 10% heat-inactivated fetal bovine serum in 5% CO_2 at 37 ℃. Cells were seeded at 9 × 10^5 cells/mL in 60-mm dishes and then treated with IL-6 (25 ng/mL) and the indicated concentrations of butein.

Colic epithelial cell isolation

Colonic epithelial cells were isolated as previously described[25]. Mouse colonic tissues were washed using cold PBS and opened longitudinally. Colons were irrigated with cold Ca^{2+}- and Mg^{+}-free Hank's balanced salt solution (CMF-HBSS). The tissue was then transferred to 5 mL CMF-HBSS containing 10 mmol/L dithiothreitol (1:100; Sigma-Aldrich Inc.) and 50 nmol/L calyculin A (1:200; Wako, Richmond, VA, United States) and incubated in rotator for 30 min at 4 ℃. The tissue was then transferred to another 5 mL CMF-HBSS solution containing 1 mmol/L ethylenediaminetetraacetic acid and 50 nmol/L calyculin A, and incubated at 4 ℃ for 1 h. Colonic tissues were removed from the tube, and epithelial cells were isolated by centrifugation at 300 rpm for 5 min. The supernatant was removed, and the remaining cells were snap-frozen in liquid nitrogen and maintained at -70 ℃ for future analysis.

Table 1 Histologic scores

Grade 0	Normal tissue
Grade 1	One or a few multifocal mononuclear cell infiltrates in the lamina propria Minimal epithelial hyperplasia Slight to no depletion of mucus from goblet cells
Grade 2	Lesions tended to involve more of the intestine than grade 1 lesions Several multifocal, mild inflammatory cell infiltrates in the lamina propria Mild epithelial hyperplasia and mucin depletion Small epithelial erosions
Grade 3	Lesions involved a large area of the mucosa or were more frequent than grade 2 lesions Moderate inflammation with the involvement of submucosa Mixture of mononuclear cells as well as neutrophils, and crypt abscesses Moderate epithelial hyperplasia and mucin depletion Ulcers were occasionally observed
Grade 4	Lesions usually involved most of the intestinal section and were more severe than grade 3 lesions Severe inflammation including mononuclear cells and neutrophils with transmural involvement Marked epithelial hyperplasia Crypt abscesses and ulcers

Immunohistochemistry

For the proliferation assay, 1 mg 5-Bromo-2'-Deoxyuridine (BrdU; Sigma-Aldrich Inc.) was injected into mice 2 h before sacrifice. Formalin-fixed, paraffin-embedded sections were processed for immunohistochemistry. Paraffin-embedded slides were deparaffinized and hydrated. Antigen retrieval was performed using Target retrieval solution in a decloaking chamber followed by staining with antibodies against pSTAT3 (1:100), BrdU (1:200) or MMP-9 (1:00) followed by anti-rabbit or anti-mouse-HRP-labeled polymers. Sections were developed using 3,3'-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

Western blot

Protein was extracted from isolated colonic epithelial cells and Colo 205 cells using protein extraction buffer (Fisher Thermo Scientific Inc., Waltham, MA, United States) as described by the manufacturer. Extracted protein concentrations were measured using the BCA method, and 30 μg protein samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to

Table 2 Primer list

Name		Sequence
IL-6	F	AGT TGC CTT CTT GGG ACT GA
	R	TCC ACG ATT TCC CAG AGA AC
IL-1b	F	GCC CAT CCT CTG TGA CTC AT
	R	AGG CCA CAG GTA TTT TGT CG
IL-22	F	CAA CTT CCA GCA GCC ATA CA
	R	GTT GAG CAC CTG CTT CAT CA
IL-17A	F	TCC AGA AGG CCC TCA GAC TA
	R	AGC ATC TTC TCG ACC CTG AA
IFN-γ	F	ACT GGC AAA AGG ATG GTG AC
	R	TGA GCT CAT TGA ATG CTT GG
MMP-2	F	CAG ACT TGT CCC GTT TCC AT
	R	GGT GCT GAC TGC ATC AAA GA
MMP-9	F	CGT CGT GAT CCC CAC TTA CT
	R	AAC ACA CAG GGT TTG CCT TC
IL-21	F	GGC AAC ATG GAG AGG ATT GT
	R	AAG CAG GAA AAA GCT GAC CA
IL-23R	F	CAT GAC TTG CAC CTG GAA TG
	R	GCT TGG ACC CAA ACC AAG TA
IL-12β (P.40)	F	AGG TCA CAC TGG ACC AAA GG
	R	TGG TTT GAT GAT GTC CCT GA

IFN: Interferon; MMP: Matrix metalloproteinase; F: Forward; R: Reverse; IL: Interleukin.

nitrocellulose membranes and blocked with Tris-buffered saline containing 0.05% Tween-20, 5% nonfat milk and 5% fetal bovine serum. The primary antibodies were applied overnight at 4 ℃. Antibodies against STAT3 (1:1000) and GAPDH served as the sample loading controls. After extensive washing, the membranes were incubated for 1 h in corresponding horseradish peroxidase-coupled secondary antibodies (donkey anti-rabbit 1:1000). The chemiluminescence reaction was developed using a West Pico reagent (Pierce, Rockford, IL, United States).

RNA isolation and quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify the expression of mRNA for cytokines and MMP-9 with the expression of GAPDH for endogenous control. Total RNA from the proximal colon of each mouse was extracted using Trizol (Invitrogen of Thermo Fisher Scientific Inc.), and cDNA was synthesized using the iScript™ cDNA synthesis Kit (Roche, Basel, Germany). qRT-PCR was performed using the LightCycler 480 SYBR Green I Master kit (Roche) according to the manufacturer's instructions with the primers summarized in Table 2.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or the Mann-Whitney *U* test. Results are expressed as mean ± SE.

RESULTS

Butein treatment ameliorates colitis in IL-10$^{-/-}$ mice

It has been established that induction with piroxicam for 2 wk accelerates and synchronizes the onset and severity of colitis in IL-10$^{-/-}$ mice with marked transmural inflammation and ulceration in the proximal colon[8,23]. During the colitis experiment, mice treated with butein exhibited a greater body weight gain and longer colonic length (Figure 2A and B). Histologic analysis demonstrated that treatment with butein (1 mg/kg) for 2 wk after cessation of the 2-wk piroxicam administration significantly reduced the inflammatory score with no visible deep ulceration and lessened inflammatory cell infiltrates (Figure 2C and D).

Butein treatment inhibits inflammatory cytokines and MMP-9 in the colons of IL-10$^{-/-}$ mice

Previous studies have shown that colitis in IL-10$^{-/-}$ mice is induced by dysregulation of Th1-mediated cytokines, including IL-1β, IFN-γ and IL-6. The Th1 cytokines, IFN-γ, IL-1β, IL-6, IL-21, IL-23 and IL-12β were heavily upregulated in IL-10$^{-/-}$ mice, together with significantly increased MMP-9, two weeks after the last administration of piroxicam. IL-10$^{-/-}$ mice that received 2-wk-treatment with butein demonstrated significantly reduced expression of *IFN-γ*, *IL-1β*, *IL-6* and *MMP-9* mRNA in the proximal colon, while there was no effect on the expression of *IL-17a*, *IL-21*, *IL-22* and *MMP-2* (Figure 3).

Butein treatment results in reduced STAT3 and MMP-9 expression in the colons of IL-10$^{-/-}$ mice

Knowing that STAT3 is part of a major intrinsic pathway for inflammation and inflammation-associated cancers that are mediated and activated by cytokines, chemokines and other mediators including IL-6, IL-1β and macrophage colony-stimulating factor, we investigated STAT3 activity in the colons of IL-10$^{-/-}$ mice. Increased STAT3 activity was noted in inflamed colonic epithelial cells, which was inhibited by butein treatment, as noted both in immunohistochemistry and Western blots (Figure 4).

Butein treatment suppressed MMP-9 expression, which was noted in adjacent inflammatory cells in mice with colitis and ulcerations in control mice. To evaluate proliferation, a BrdU incorporation assay was performed by immunohistochemical analysis. There was no significant difference between the control and butein treatment groups (Figure 5).

Butein inhibits STAT3 phosphorylation induced by IL-6 in human Colo 205 cells

We investigated the effects of butein on the modulation of IL-6/STAT3 activation *in vitro* using Colo 205 cells. Exposure to IL-6 for different times and at different concentrations increased phosphorylation of STAT3. Butein treatment suppressed the phosphorylation of STAT3 induced by IL-6 (25 ng/mL) at a concentration of 10 μmol/L (Figure 6).

DISCUSSION

The objective of this study was to examine whether

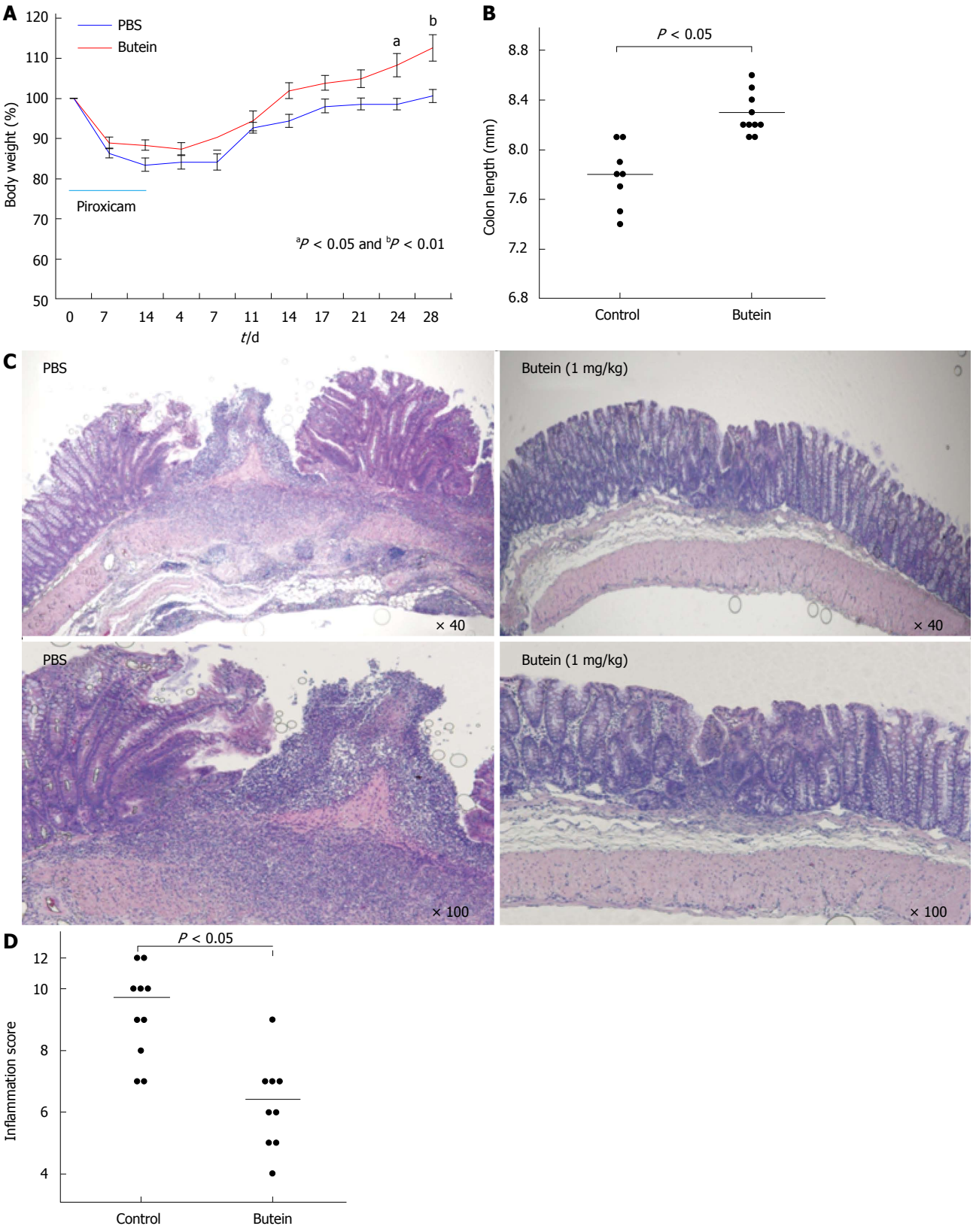

Figure 2 Butein treatment ameliorates colitis. A and B: Body weight and colon length between the two groups revealed a significant difference; C: Hematoxylin and eosin stain of Swiss rolls showing ulcers and inflammation; D: Histologic score of mice treated with butein or phosphate-buffered saline (n = 9 per group). PBS: Phosphate-buffered saline.

butein, a naturally derived substance, had therapeutic effects in IL-10$^{-/-}$ mice, an IBD model. The occurrence of IBD significantly decreased in mice injected with butein; butein blocked the IL-6/STAT3 signal trans-

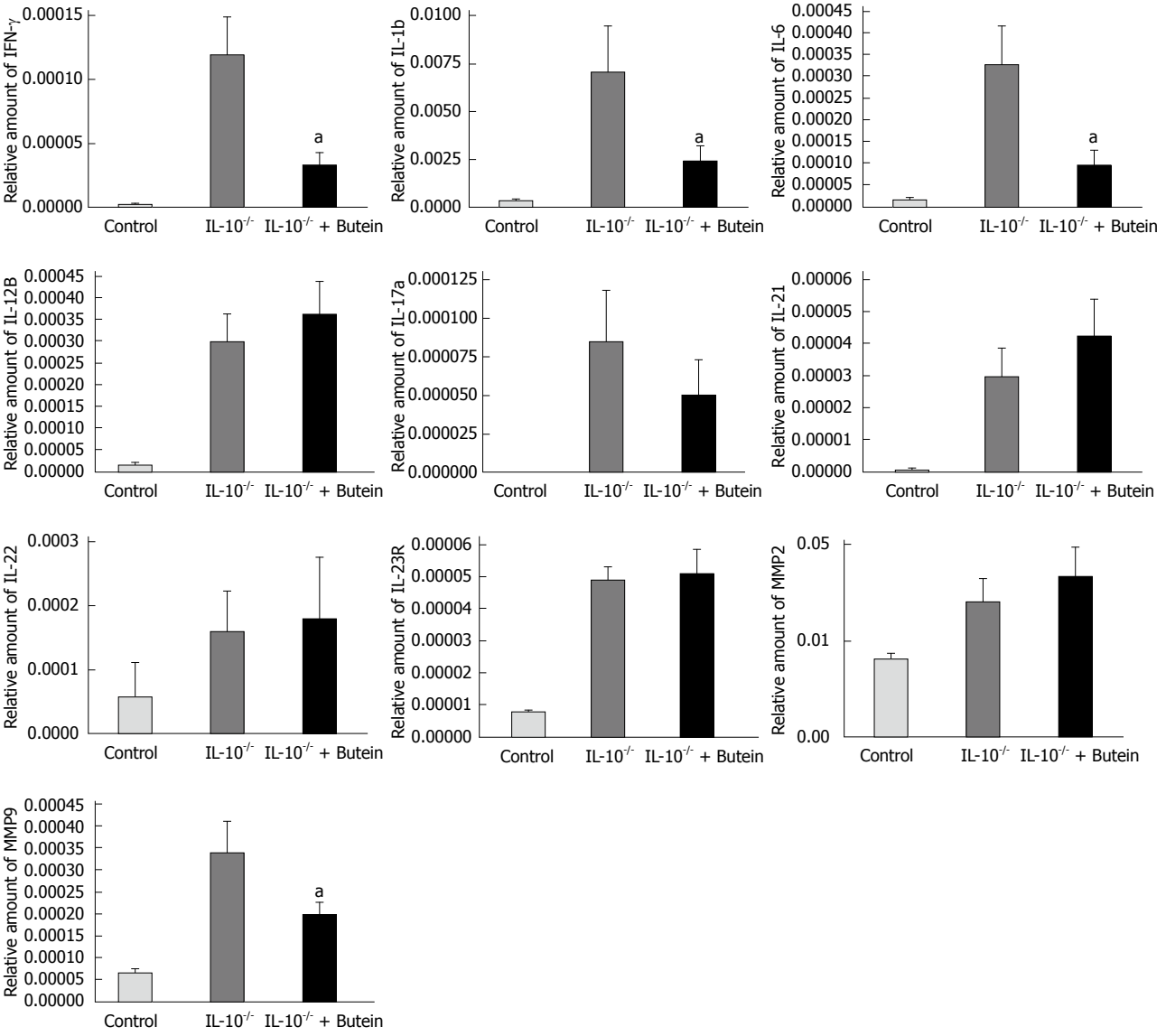

Figure 3 Expression of cytokines and matrix metalloproteinases in the colons of interleukin-10$^{-/-}$ mice. Relative expression levels of mRNA were determined by real-time polymerase chain reaction. Data are expressed as mean ± SE; n = 5 per group; $^{a}P < 0.05$ *vs* control. IL: Interleukin.

mission pathway and suppressed MMP-9 activation. Butein, a major constituent of *Toxicodendron vernicifluum*, can also be found in the stems of *Semecarpus anacardium* and in the heartwood of *Dalbergia odorifera*, as well as other plants. Previous studies have reported that butein has anti-oxidant, anti-inflammatory and antitumor effects, and that it suppresses angiogenesis[26-29]. Butein is known to suppress cell proliferation and promote apoptosis in both solid and hematologic tumors; it is also less toxic than urushiol, another constituent of *Toxicodendron vernicifluum*[30,31]. Butein's effects on tumor cells arise as a result of suppressing c-Src and JAK1/JAK2 activation, thus inhibiting the IL-6/STAT3 pathway. Butein also directly inhibits the expression of Bcl-xL, Bcl-2 and cyclin D1, the target genes of STAT3[31]. STAT3 plays an important role in cytokine receptor transmission, which is a system that connects a membrane receptor to nuclear transcription and is principally activated by gp130-related cytokines, the most representative of which is IL-6.

STAT3, a principal mechanism of the inflammatory reaction and inflammation-related malignant tumors, is involved in the inflammatory reaction and inflammation-related malignant changes from the initial stage to tumor progression. STAT3 modulates the activity of NF-κB and is activated by the IL-6/JAK signal pathway[32-34]. The IL-6/JAK/STAT3 signal system promotes tumorigenesis by inducing cellular or epigenetic changes that follow intracellular inflammation. IL-6/JAK/STAT3, activated by gp130-related IL-6, IL-22, cytokines, and other growth factors, is found to be active in multiple malignant tumors, such as multiple myeloma, lymphoma, hematologic malignancies, breast cancer and prostate cancer.

In this study, *IL-6* mRNA expression was increased in IL-10$^{-/-}$ mice with bowel inflammation, and STAT3 activation was also observed in colonic epithelial cells with inflammation and ulcers following the inflammation. Butein inhibited the expression of STAT3 in epithelial

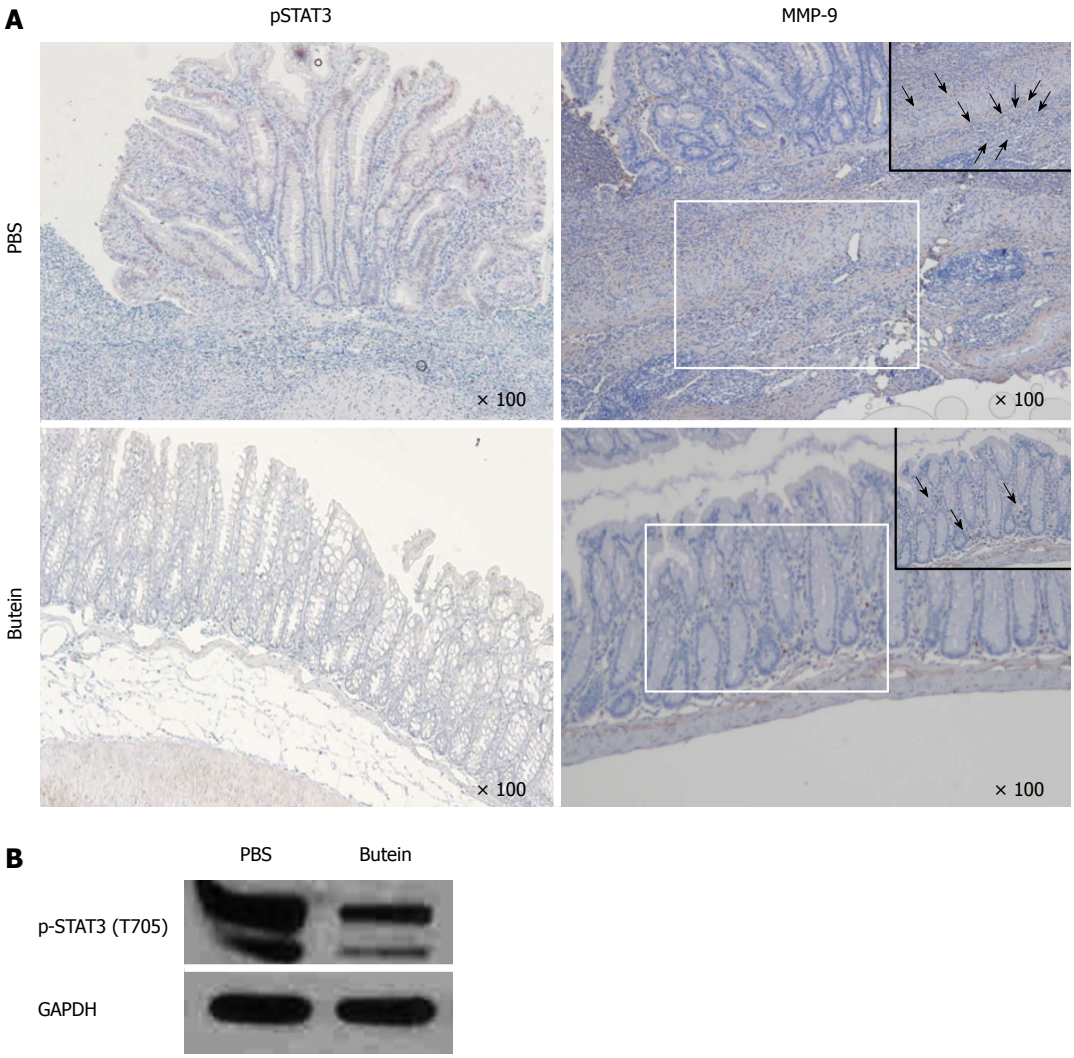

Figure 4 Butein treatment decreased mucosal expression of phosphorylated signal transducer and activator of transcription 3 and matrix metalloproteinase-9 expression in interleukin-10$^{-/-}$ mice. A: Immunohistochemical staining for phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and matrix metalloproteinase (MMP)-9 detected nuclei of epithelial cells or stromal cells of interleukin (IL)-10$^{-/-}$ mice (black arrows); B: Western blot analysis revealed reduced pSTAT3 protein expression in intestinal epithelial cells of butein-treated IL-10$^{-/-}$ mice (n = 3). PBS: Phosphate-buffered saline.

cells, which was demonstrated by immunohistochemical staining and Western blot analysis. We also found that MMP-9 expression in the colonic tissues was blocked by butein. *In vitro* experiments showed that IL-6-activated MMP-9 was highly concentrated, and this activity was blocked by butein. MMP-9 is closely connected with tissue remodeling and tumor metastasis, and is secreted with MMP-2 from tumor cells, inflammatory cells and cell matrix cells[35]. In a previous *in vitro* study, butein was reported to inhibit the activation of MMP-9[36]. Similarly, we found that butein blocked the increased expression of MMP-9 in inflammatory cells, and infiltration of the muscle layer in bowel inflammation or inflammation-induced ulcers.

Here, we present the first *in vivo* study examining the therapeutic effects of butein in an animal model of bowel inflammation. There were a few limitations to our study. First, there was no confirmatory analysis of MMP-9 with zymography for analyzing MMP-9 activation after butein treatment. Second, we did not determine whether the protein expression of IL-6 matched mRNA levels. Third, we had other limitations related to our experiment methods. These shortcomings will be further modified in future studies. Finally, no quantitative analysis of inflammatory cytokine activation was undertaken, and the analysis of the signal transmission system was limited to epithelial cells. It is reasonable to state that IL-10$^{-/-}$ mice are not an appropriate model to study the proliferation and recovery of epithelial cells, as they are a model principally defined by the degree of inflammatory cytokine expression. This limitation could be overcome by conducting additional experiments with other study models. In doing so, the therapeutic effects of butein could be evaluated more accurately.

The results of this study regarding the interfering effects of butein on the IL-6/STAT3-MMP-9 pathway are similar to those of an *in vitro* study that used established malignant tumor cells. Considering that the suppression of bowel inflammation and the recovery capability of the injured mucous membrane are critical

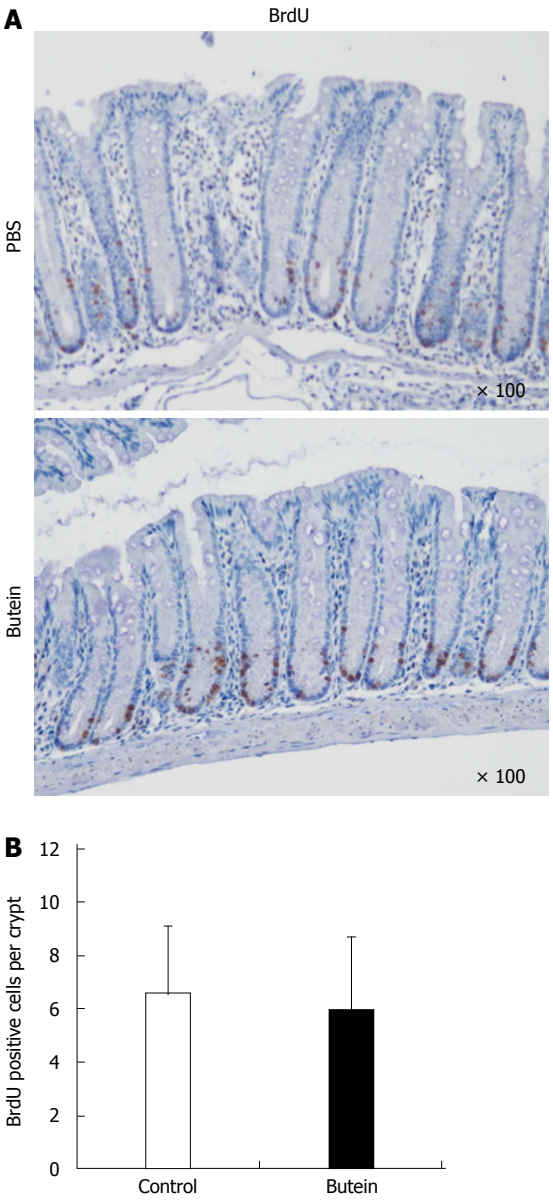

Figure 5 Immunohistochemistry for BrdU incorporation. A and B: Immunohistochemisty for BrdU incorporation revealed no significant difference in proliferation of the intestinal epithelial cells between the two groups. PBS: Phosphate-buffered saline.

for IBD treatment, the findings obtained from the BrdU analysis indicating that the cell proliferation necessary for the recovery of the mucous is not affected by butein treatment further supports the clinical applicability of butein in treating IBD.

Chronic inflammation is a major mechanism of malignant tumors. The incidence of malignant tumors in the colon and the small intestine is significantly increased with IBD such as ulcerative colitis and CD. The IL-6/STAT3 pathway is an important pathway for the initiation of inflammation-mediated malignant tumors, and MMP-9 is an essential signal transmission system related to the metastasis of malignant tumors. In IBD patients, the expression of IL-6 is increased, and the expression of MMP-9 is known to serve a critical role in pathogenesis.

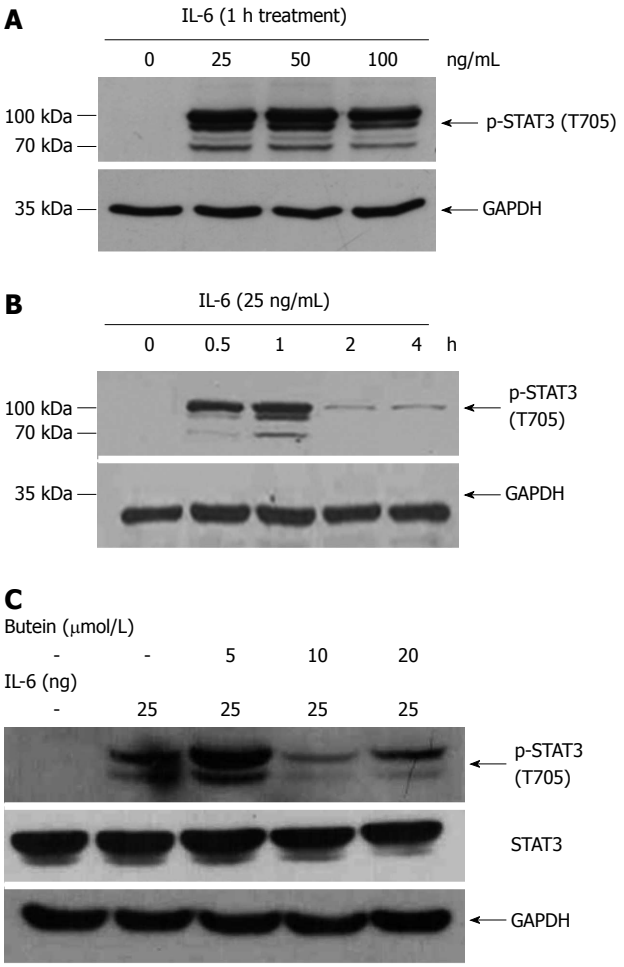

Figure 6 Butein inhibited the interleukin-6-induced activation of signal transducer and activator of transcription 3 in Colo 205 cells. A: Colo 205 cells were treated with various concentrations of interleukin (IL)-6; B: Colo 205 cells were treated with IL-6 for various times; C: Expression levels of phosphorylated signal transducer and activator of transcription 3 (pSTAT3), were measured by Western blot analysis after pretreating Colo 205 cells with IL-6 (25 ng/mL) for 1 h prior to butein treatment for 24 h at various concentrations (n = 3).

Our results point to the possibility of applying butein to bowel inflammation-induced colon cancer, as butein suppressed bowel inflammation and interfered with the IL-6/STAT3 and MMP-9 pathways in IL-10$^{-/-}$ mice. It is therefore important to further investigate the effects of butein on the occurrence of bowel inflammation-related colon cancer.

COMMENTS

Background

The therapeutic effects of natural substances on inflammation and tumors are being widely studied. Butein has been found in *in vitro* studies to have antioxidant and anti-inflammatory effects and to suppress tumor cell proliferation and angiogenesis.

Research frontiers

The suppressive effects that butein has on matrix metalloproteinase-9 (MMP-9) activation have been reported in an *in vitro* study using several cancer cells. However, no study has yet to be conducted that examines the therapeutic effects of butein on bowel inflammation and colon cancer. This study, therefore,

aims to evaluate the interfering effects of butein on inflammatory cytokines and MMP-9 in interleukin (IL)-10$^{-/-}$ mice, an inflammatory bowel disease model, and ultimately to examine the therapeutic effects of butein.

Innovations and breakthroughs

The results suggest that butein could be used to treat bowel inflammation-induced colon cancer, as it suppressed bowel inflammation and interfered with the IL-6/signal transducer and activator of transcription 3 (STAT3) and MMP-9 pathways in IL-10$^{-/-}$ mice. To our knowledge, while there have been several *in vitro* studies on tumor cells, this is the first *in vivo* study to show the effect of butein in mice with colitis.

Applications

The study results suggest that it is important to represent the inhibitory effects of butein on the occurrence of bowel inflammation-related colon cancer by regulating IL-6/STAT3 and MMP-9 activation.

Peer review

The authors examined whether butein, a naturally derived substance, had therapeutic effects in IL-10$^{-/-}$ mice, a model of inflammatory bowel disease. Butein blocked the IL-6/STAT3 signal transmission pathway and suppressed MMP-9 activation. The results are interesting and may represent the effects of butein on the occurrence of bowel inflammation-related colon cancer.

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ORIGINAL ARTICLE

Basic Study

Chemokine ligand 20 enhances progression of hepatocellular carcinoma *via* epithelial-mesenchymal transition

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Abstract

AIM: To identify the mechanisms of chemokine ligand 20 (CCL20)-induced hepatocellular carcinoma (HCC) metastasis and evaluate it as a prognostic marker.

METHODS: Expression of CCL20 was evaluated by immunohistochemistry in HCC tissues from 62 patients who underwent curative resection. The relationship between CCL20 expression and clinicopathologic features was analyzed. Univariate and multivariate analyses were performed to evaluate its predictive value for recurrence and survival of HCC patients. The expression levels of epithelial-mesenchymal transition (EMT)-and signaling pathway-related proteins were evaluated by Western blotting and immunocytochemistry. The effects of CCL20 on HCC cell proliferation and migration were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT) and Transwell assays.

RESULTS: CCL20 immunoreactivity was detected in all 62 patient specimens. CCL20 expression was associated with preoperative alpha-fetoprotein level (P = 0.043), tumor size (P = 0.000), tumor number (P = 0.008), vascular invasion (P = 0.014), and tumor differentiation (P = 0.007). Patients with high CCL20 expression had poorer recurrence-free and overall survivals compared to those with low CCL20 expression (both P < 0.001). CCL20 induced EMT-like changes in HCC cells and increased their proliferation and migration ability (P < 0.05). Western blotting and immunofluorescence staining showed that CCL20 induced an EMT-like phenotype in HCC cells, and increased expression of phosphorylated AKT, β-catenin and vimentin, and decreased E-cadherin expression (P < 0.05). The correlation analysis revealed that high CCL20 expression in HCC tissue specimens was negatively correlated with E-cadherin expression (13.33%, 4/30), and positively correlated with vimentin (90.0%, 27/30), β-catenin (96.67%, 29/30) and p-AKT (76.67%, 23/30) expression.

CONCLUSION: CCL20 expression is associated with HCC recurrence and patient survival and promotes HCC cell proliferation and migration by inducing EMT-like changes *via* PI3K/AKT and Wnt/β-catenin pathways.

Key words: Chemokine ligand 20; Phosphoinositide kinase-3/AKT; Prognosis; Wnt/β-catenin; Epithelial-mesenchymal transition; Hepatocellular carcinoma

Core tip: This study examined the expression and prognostic value of chemokine ligand 20 in hepatocellular carcinoma. The results indicate that increased expression of this protein regulates the growth and migration of hepatocellular carcinoma cells and epithelial-mesenchymal transition *via* phosphoinositide kinase-3/AKT, and Wnt/β-catenin signaling pathways and is therefore a potential treatment target.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of cancer death worldwide[1-3]. HCC is associated with a poor outcome and a high rate of mortality due to the high rate of recurrence and metastasis[4-6]. Thus, it is essential to identify novel predictors of recurrence and metastasis. The risk factors for HCC include hepatitis B or C virus infection, alcohol consumption, and non-alcoholic fatty liver disease[7-10]. Moreover, the tumor microenvironment contains various cytokines, chemokines and growth factors that are produced by tumor or stromal cells, which promote tumor initiation, progression and metastasis[11,12].

Chemokine ligand 20 (CCL20), also known as liver activation regulated chemokine or macrophage inflammatory protein-3, is a small cytokine that is strongly chemotactic for lymphocytes and weakly attracts neutrophils[13]. Increasing evidence indicates that CCL20 is related to tumor formation, progression or metastatic processes in many malignancies, including breast and colorectal cancer[14,15]. The proliferation and migration of tumor cells are considered a foundation of cancer survival and development. Tumor progression and metastasis are associated with the induction of epithelial-mesenchymal transition (EMT)[16,17]. In this study, CCL20 expression was examined in HCC patient samples and the relationships with clinicopathologic features and recurrence and patient survival were examined. Moreover, the effects of CCL20 expression on HCC cell EMT, proliferation and migration and involvement of relevant signaling pathways were investigated.

MATERIALS AND METHODS

Patients and specimens

From January 2002 to October 2008, 62 consecutive patients with primary HCC who underwent radical resection at our hospital were enrolled in the study. The diagnosis was confirmed by histologic examination in all cases. All primary tumor tissues were preserved in paraffin for immunohistochemical analyses. None of the patients received preoperative anticancer treatment. Preoperative liver function was evaluated using the Child-Pugh scoring system. Tumor stage was determined according to the tumor-node-metastasis (TNM) classification system of the American Joint Cancer Committee/Union for International Cancer Control (2002). Tumor differentiation was graded using the Edmondson-Steiner classification system. Data was censored at the last follow-up for patients without recurrence or death. Recurrence-free survival (RFS) and overall survival (OS) was defined as the interval between the time of surgery to that of recurrence or death, respectively. This study was approved by the Ethics Committee of our hospital. Written informed consent was obtained from all patients.

Cell culture and treatment

Human HCC cell lines Hep3B and Huh7 were maintained in Dulbecco's Modified Eagle's Medium (Gibco of Thermo Fisher Scientific Inc., Waltham, MA, United States) supplemented with 10% fetal calf serum and 100 IU/mL penicillin and 100 mg/mL streptomycin in a 5% CO_2 incubator at 37 ℃. CCL20 (Cat. 360-MP-025; R&D Systems, Minneapolis, MN, United States) was added to the media, which was changed every other day.

Cell proliferation assay

Cell proliferation was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT) assay. Cells were plated in 96-well culture plates at 5×10^3 cells per well containing 0.2 mL of culture media. After treatment with CCL20 (5 μg/mL) for 24 or 48 h, 0.02 mL of 5 mg/mL MTT was added to each well and incubated for 4 h at 37 ℃. The absorbance was measured at 570 nm. Each assay was performed three times independently.

Invasion assay

Cell invasion was assessed using Transwell chambers (8 μm pore size; EMD Millipore, Billerica, MA, United States) according to the manufacturer's instructions. ECM (Sigma-Aldrich, St. Louis, MO, United States) was added to the chamber to form a gel layer, and cells (5×10^4) were added to the upper chamber in the presence of CCL20 at a concentration of 5 μg/mL for 24 or 48 h. The cells migrating to the membrane were enumerated with Giemsa staining. The assay was performed three times independently.

Primary antibodies

The following primary antibodies used in the experiments were obtained from Santa Cruz Biotechnologies Inc., Dallas, TX, United States: anti-E-cadherin (sc-21791), anti-vimentin (sc-53464), anti-AKT (sc-5298), anti-phospho(p)-AKT (sc-33437), anti-β-catenin (sc-7963) and anti-GAPDH (sc-25778).

Table 1 Relationship between chemokine ligand 20 expression and clinicopathologic features

Clinicopathologic features	Number of patients	CCL20 expression		*P* value
		Low (n = 32)	High (n = 30)	
Age (yr)				
≤ 57	35	19	16	0.632
> 57	27	13	14	
Gender				
Male	47	26	21	0.301
Female	15	6	9	
Etiology				0.167
HBV infection	46	26	20	
HCV infection	11	2	9	
Alcohol	5	4	1	
Background liver pathology				0.122
Normal liver	3	2	1	
Chronic hepatitis	20	11	9	
Liver cirrhosis	39	19	20	
AFP (ng/mL)				
≤ 197	33	21	12	0.043
> 197	29	11	18	
ALT (U/L)				
≤ 66	28	17	11	0.193
> 66	34	15	19	
Child-Pugh				
A	44	21	23	0.338
B	18	11	7	
Tumor size (cm)				
≤ 5	41	29	12	0.000
> 5	21	3	18	
Tumor number				
Single	43	27	16	0.008
Multiple	19	5	14	
Vascular invasion				
No	51	30	21	0.014
Yes	11	2	9	
Tumor encapsulation				
Yes	24	14	10	0.400
No	38	18	20	
Tumor differentiation				
Ⅰ + Ⅱ	45	28	17	0.007
Ⅲ + Ⅳ	17	4	13	
TNM stage				
Ⅰ	40	22	18	0.472
Ⅱ + Ⅲ	22	10	12	

AFP: Alpha fetoprotein; CCL20: Chemokine ligand 20; HBV: Hepatitis B virus; HCV: Hepatitis C virus; OS: Overall survival; RFS: Recurrence-free survival; TNM: Tumor-node-metastasis; ALT: Alanine aminotransferase.

Western blotting analysis

Lysates were extracted from cells treated with CCL20 at a concentration of 5 μg/mL for 48 h using lysis buffer with phenylmethylsulfonyl fluoride, and the protein concentration was measured with a BCA protein assay kit (Beyotime, Shanghai, China). Total protein extracts (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (20 mmol/L Tris-HCl, 150 mmol/L NaCl and 0.1% Tween-20, pH 7.5) for 1 h at room temperature and individually incubated overnight at 4 ℃ with antibodies against E-cadherin (1:1000), vimentin (1:1000), AKT (1:1000), p-AKT (1:1000), β-catenin (1:1000) or GAPDH (1:5000). Signals were detected by using enhanced ECL chemiluminescence (MultiScience Biotech Co., Shanghai, China) and analyzed using Quantity One (Bio-Rad, Hercules, CA, United States).

Immunohistochemistry

Immunohistochemical detection of E-cadherin (1:100), vimentin (1:500), p-AKT (1:100) and β-catenin (1:100) was performed on 4 μm-thick sections of specimens which had been fixed in formalin, embedded in paraffin and mounted on slides. Replacement of the primary antibody with mouse- or rabbit-isotype control antibody served as a negative control. Counterstaining of the nucleus was performed using hematoxylin. The staining intensity was scored in four levels (0 = negative, 1 = weak, 2 = moderate, 3 = strong), and the percentages of stained cells at each intensity level were counted. The total immunostaining score was calculated as the sum of each intensity score multiplied by the corresponding percentage. The slides were independently evaluated and scored by three pathologists without knowledge of clinical data.

Immunocytochemistry

After reaching confluency, cells plated on coverslips in 6-well dishes were washed twice, fixed with 2% (w/v) formaldehyde and permeabilized with 1% (v/v) Triton X-100. Coverslips were blocked with 10% (w/v) normal goat serum in phosphate-buffered saline at room temperature for 1 h and then incubated in primary antibodies against E-cadherin (1:200), vimentin (1:500), p-AKT (1:200) or β-catenin (1:400) at 4 ℃ overnight. Cells were washed and incubated with Cy3-labeled secondary antibody (Beyotime) at room temperature for 1 h, and co-stained with DAPI (Sigma-Aldrich) to visualize nuclei. Images were obtained using a fluorescence microscope at magnification × 200.

Statistical analyses

All analyses in the study were performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, United States). One-way analyses of variance and Student's *t*-tests were used for intergroup comparisons. The correlation between CCL20 expression and clinicopathologic features was examined by the χ^2 test. RFS and OS were calculated by the Kaplan-Meier method. Univariate analyses for factors of recurrence and survival were performed using the χ^2 test and the log-rank test, respectively. Data are presented as mean ± SD, with $P < 0.05$ considered as statistically significant.

RESULTS

Clinical data and follow-up

Of the HCC patient samples, 75.8% (47/62) were from men and 24.2% (15/62) were from women (Table 1). The average age of the patients was 57 years (range:

Table 2 Univariate analyses for factors associated with recurrence and survivals

Factor	Recurrence		*P* value	RFS	*P* value	OS	*P* value
	Yes	No		(%)		(%)	
Age (yr)							
≤ 57	19	16	0.184	53.2	0.211	66.7	0.135
> 57	18	9		38.1		50.6	
Gender							
Male	26	21	0.469	58.9	0.192	55.4	0.633
Female	9	6		42.3		64.8	
Etiology			0.235		0.659		0.835
HBV infection	27	19		45.7		56.3	
HCV infection	7	4		39.2		54.5	
Alcohol	2	3		58.4		61.7	
Background liver pathology			0.953		0.784		0.947
Normal liver	2	1		55.3		63.5	
Chronic hepatitis	13	7		45.6		57.4	
Liver cirrhosis	22	17		40.9		50.2	
AFP (ng/mL)							
≤ 197	15	18	0.084	58.3	0.038	65.5	0.074
> 197	19	10		29.6		42.8	
ALT (U/L)							
≤ 66	15	13	0.371	45.6	0.881	55.3	0.942
> 66	19	15		41.5		52.9	
Child-Pugh							
A	24	20	0.774	40.2	0.695	54.1	0.133
B	11	7		38.8		44.3	
Tumor size (cm)							
≤ 5	21	20	0.417	52.9	0.024	68.3	0.014
> 5	14	7		31.7		44.8	
Tumor number							
Single	25	18	0.217	47.1	0.052	58.3	0.233
Multiple	13	6		30.2		46.5	
Vascular invasion							
No	31	20	0.042	54.6	0.019	62.8	0.008
Yes	8	3		31.8		40.9	
Tumor encapsulation							
Yes	14	10	0.649	47.6	0.443	65.3	0.032
No	23	15		38.2		43.1	
Tumor differentiation							
Ⅰ + Ⅱ	21	24	0.241	45.3	0.695	59.6	0.047
Ⅲ + Ⅳ	11	6		41.8		40.1	
TNM stage							
Ⅰ	19	21	< 0.001	56.8	< 0.001	66.4	< 0.001
Ⅱ + Ⅲ	18	4		20.4		35.7	
CCL20							
Low	13	19	< 0.001	60.9	< 0.001	70.1	< 0.001
High	27	3		21.4		30.6	

AFP: Alpha fetoprotein; ALT: Alanine aminotransferase; CCL20: Chemokine ligand 20; HBV: Hepatitis B virus; HCV: Hepatitis C virus; OS: Overall survival; RFS: Recurrence-free survival; TNM: Tumor-node-metastasis.

32-79 years), with 91.9% (57/62) of the patients having Hepatitis B or C viral infections, and 95.2% (59/62) presenting with chronic hepatitis history or liver cirrhosis. The average values of alpha-fetoprotein (AFP) and alanine aminotransferase before surgery were 197 ng/ml and 66 U/L, respectively. TNM stages of all HCC samples were divided into a stage Ⅰ group (40/62; 64.5%) or a stage Ⅱ and Ⅲ group (22/62; 35.5%). The average follow-up period was 42.6 ± 19.8 mo (range: 8-65 mo), and 66.1% (41/62) of patients presented recurrence after surgery. The recurrence sites included the liver (n = 32), lung (n = 4), lymph node (n = 5) and bone (n = 2). OS and RFS were 88.7% (55/62) and 83.9% (52/62) at 1 year, 74.2% (46/62) and 66.1% (41/62) at 3 years, and 53.2% (33/62) and 33.9% (21/62) at 5 years, respectively.

Correlation between CCL20 expression and clinicopathologic factors

The expression of CCL20 was examined in all 62 HCC samples, and was mainly located in the cytoplasm of HCC cells (Figure 1). The average immunohistochemistry score was 165.0 (range: 85-260). All HCC samples were subsequently divided into low CCL20 group (n = 32) and high CCL20 group (n = 30) by using the average value. The relationships between CCL20 expression and clinicopathologic factors are presented in Table 1. Our findings revealed that CCL20 expression was significantly related to preoperative AFP level (P = 0.043), tumor size (P = 0.000), tumor number (P = 0.008), vascular invasion (P = 0.014), and tumor differentiation (P = 0.007).

Correlations between CCL20 expression and HCC recurrence and patient survival

Univariate analyses indicated that HCC recurrence was related with vascular invasion (P = 0.042), TNM stage (P < 0.001), and CCL20 expression (P < 0.001) (Table 2). RFS was significantly related with preoperative AFP (P = 0.038), tumor size (P = 0.024), vascular invasion (P = 0.019), TNM stage (P < 0.001), and expression of CCL20 (P < 0.001). OS significantly related with tumor size (P = 0.014), vascular invasion (P = 0.008), tumor encapsulation (P = 0.032), tumor differentiation (P = 0.047), TNM stage (P < 0.001), and CCL20 expression (P < 0.001). There were no significant correlations among the other clinicopathologic factors and recurrence or survivals.

CCL20 promotes in vitro proliferation and invasion of HCC cells

The proliferation of Hep3B and Huh7 HCC cells was significantly enhanced by CCL20 after 24 and 48 h (both P < 0.05) (Figure 2A). Similarly, results of the invasion assay indicated that CCL20 treatment significantly increased invasion of Hep3B and Huh7 HCC cells (both P < 0.05) (Figure 2B).

CCL20 induces EMT-like phenotype and activates PI3K/AKT and Wnt/β-catenin pathways in HCC cells

Western blotting results showed that CCL20 at a concentration of 5 μg/mL for 48 h induced an EMT-like phenotype in Hep3B and Huh7 HCC cells. The expression of the epithelial marker E-cadherin was decreased and the mesenchymal marker vimentin was increased significantly by CCL20 (P < 0.05) (Figure 3). Moreover, β-catenin levels and phosphorylation of AKT were also increased compared with controls (P < 0.05).

Treatment with CCL20 (5 μg/mL) for 48 h induced an EMT-like phenotype in Hep3B and Huh7 cells (Figure 4A). The detection of β-catenin and p-AKT by

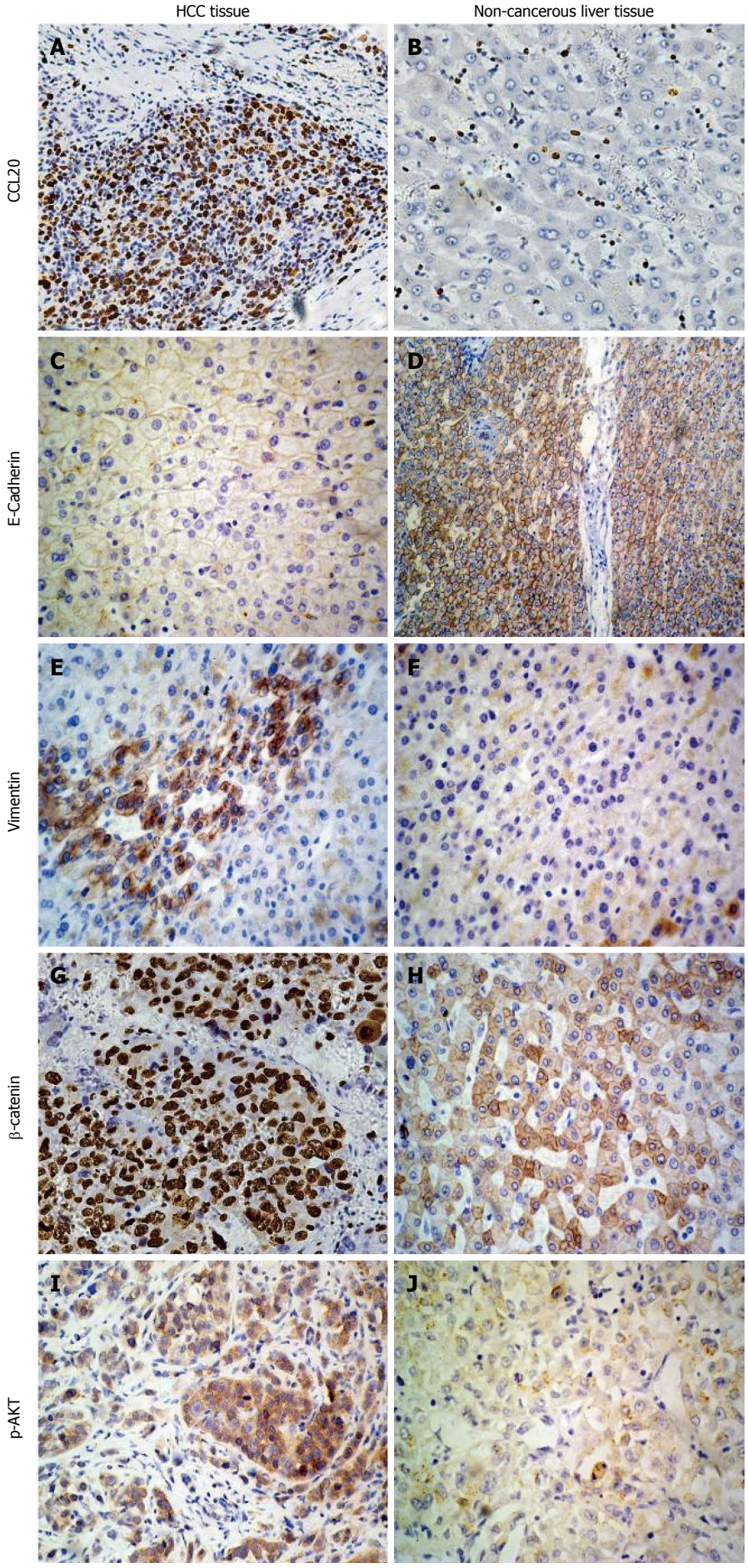

Figure 1 Immunohistochemical analysis of hepatocellular carcinoma tissues. Expression of A, B: Chemokine ligand 20 (CCL20); C, D: E-cadherin; E, F: Vimentin; G, H: β-catenin; I, J: Phosphorylated AKT (p-AKT) in HCC and non-cancerous liver tissues (magnification × 200). HCC: Hepatocellular carcinoma.

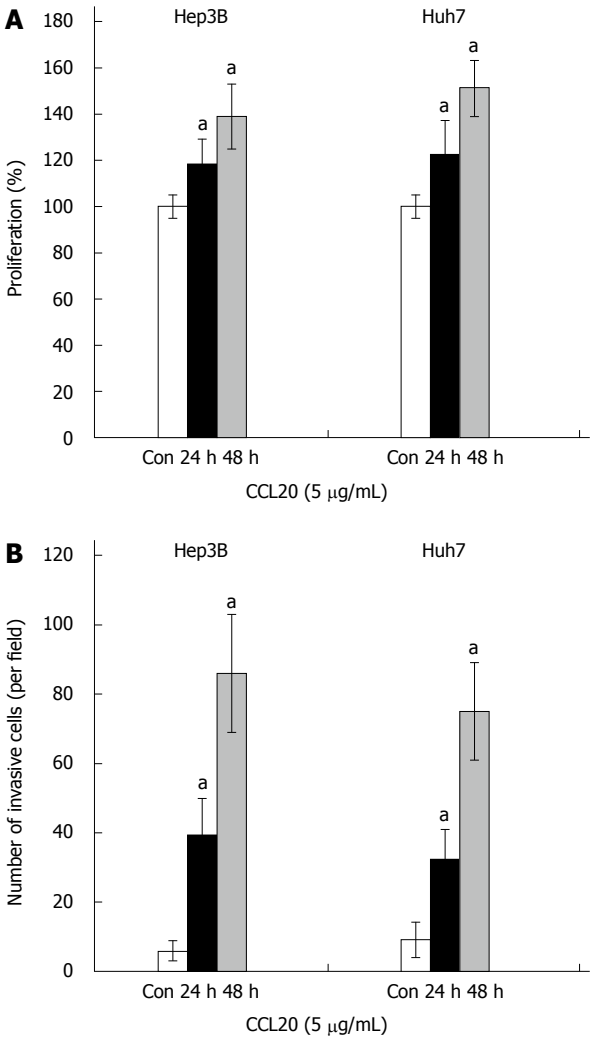

Figure 2 Effects of chemokine ligand 20 on proliferation and invasion of hepatocellular carcinoma cells. Hep3B and Huh7 cells were treated with chemokine ligand 20 (CCL20) (5 μg/mL) for 24 h and 48 h. A: Cell proliferation was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT assay); B: Invasion was assessed using a Transwell chamber; $^{a}P < 0.05$ *vs* Control. Con: Control.

immunofluorescence was enhanced by CCL20 treatment (Figure 4B).

High expression of CCL20 is associated with the changes of EMT markers in HCC specimens

EMT markers E-cadherin and vimentin, and cell-signaling pathway-related proteins β-catenin and p-AKT were also measured by immunohistochemistry in patient specimens (Figure 1). Results showed that vimentin, β-catenin and p-AKT levels were much higher and E-cadherin level was lower in samples identified as having high CCL20 expression compared to those with low expression and non-cancerous liver tissues. The correlation analysis revealed that high-CCL20 expression was negatively correlated with E-cadherin expression (4/30; 13.33%), and positively correlated with vimentin (27/30; 90.0%),

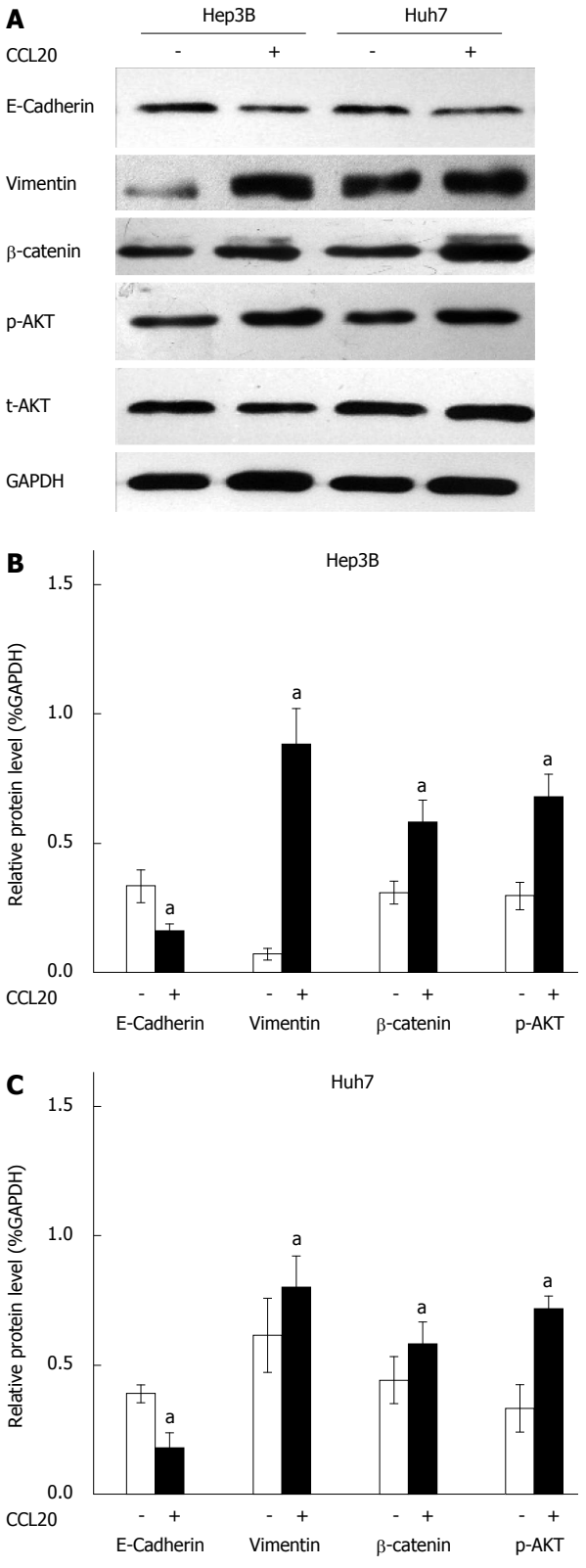

Figure 3 Effects of chemokine ligand 20 on expression of epithelial-mesenchymal transition-related proteins. A: Representative Western blots for epithelial marker E-cadherin, mesenchymal marker vimentin, total and phosphorylated AKT (t-AKT and p-AKT), and β-catenin in Hep3B and Huh7 cells after treatment with chemokine ligand 20 (CCL20) for 48 h. B: Quantification of Western blotting by gray value analysis; $^{a}P < 0.05$ *vs* Control.

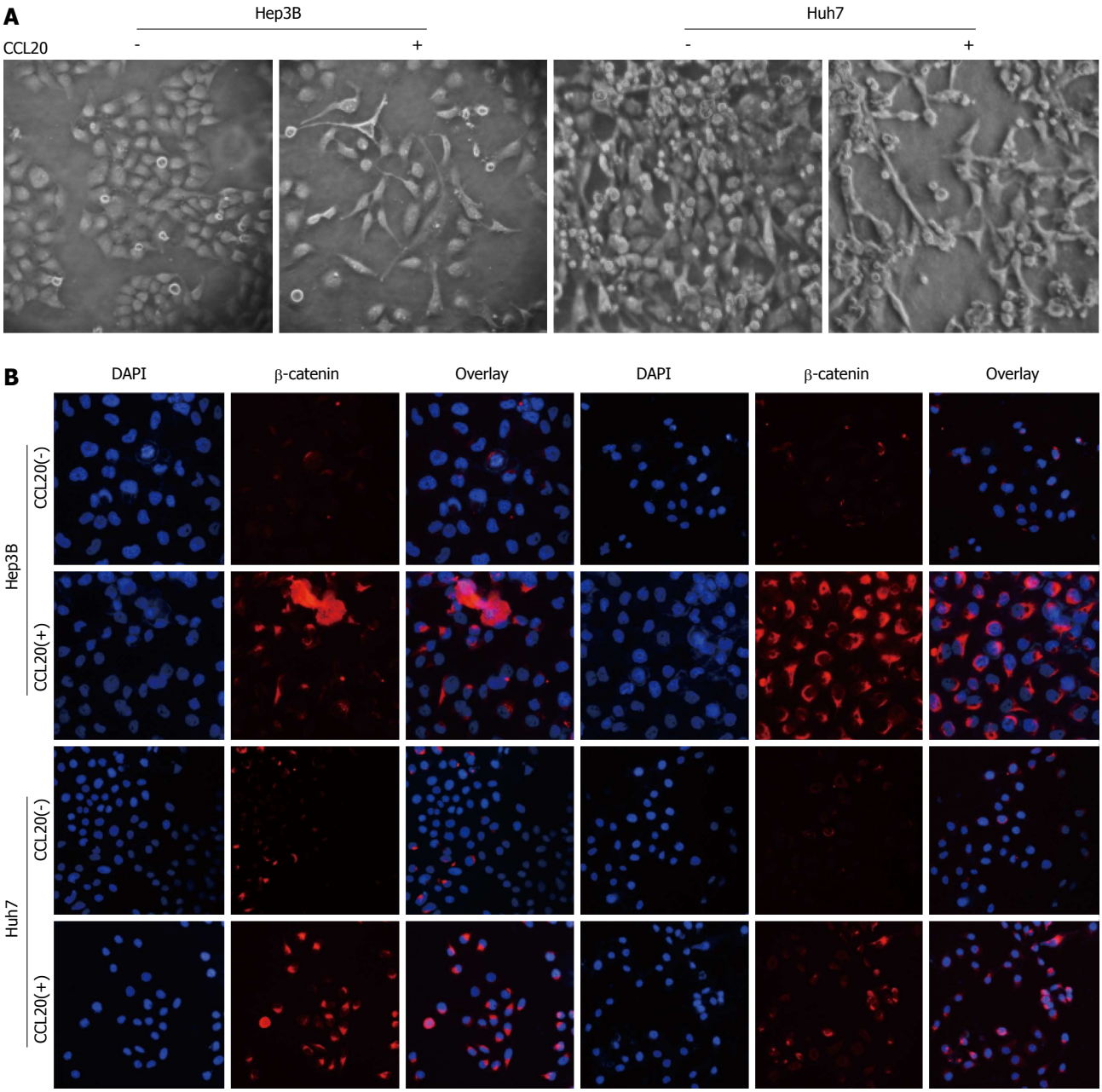

Figure 4 Epithelial-mesenchymal transition-like phenotype and upregulation of related markers in hepatocellular carcinoma cells with chemokine ligand 20. A: Morphologic changes in Hep3B and Huh7 cells after treatment with chemokine ligand 20 (CCL20) for 48 h (most cells exhibited an elongated, spindle-shape mesenchymal morphology with treatment); B: Immunocytochemistry for phosphorylated AKT (p-AKT) and β-catenin in Hep3B and Huh7 cells after treatment with CCL20 for 48 h (magnification × 200).

β-catenin (29/30; 96.67%) and p-AKT (23/30; 76.67%) expression (Table 3).

DISCUSSION

Chemokines play a critical role in various biologic events, such as the role of inflammatory responses in regulating the transfer of white blood cells, embryonic development, wound healing, angiogenesis, Th1/Th2 development, leukocyte homeostasis, and lymphatic organ development. Recent studies have suggested that chemokines and their receptors are associated with the pathogenesis, progression and metastasis of many kinds of tumors, including HCC. Li *et al*[18] found a much higher expression of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhosis, liver adenocarcinoma, and normal liver tissues. Fujii *et al*[19] performed *in vitro* experiments showing that the CCL20-CCR6 axis promotes the growth of Huh7 cells through phosphorylation of mitogen-activated protein kinase. Rubie *et al*[20] reported that CCL20 was significantly upregulated in HCC specimens. Zheng *et al*[21] found that CXCR7 expression was increased in HCC tissues and was associated with HCC invasion, adhesion and angiogenesis. Zhou *et al*[22] found that CXCL5 promotes HCC cell proliferation, invasion and intratumoral neu-

Table 3 Correlation of chemokine ligand 20 expression with different markers

	E-cadherin		Vimentin		p-AKT		β-catenin	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
CCL20	4	26	27	3	23	7	29	1
χ^2	13.285		14.754		9.589		17.796	
P value	< 0.001		< 0.001		< 0.001		< 0.001	

CCL20: Chemokine ligand 20; p-AKT: phosphorylated AKT.

trophil infiltration and could be a novel prognostic predictor of HCC.

Our results revealed that high expression of CCL20 in HCC tissues was correlated with poor outcome in HCC patients undergoing resection surgery. Our results also indicated that the expression of CCL20 was associated with tumor size, number and tumor differentiation, and with vascular invasion. Results of *in vitro* experiments indicated that CCL20 can induce EMT-like morphologic changes and promote the proliferation and invasion in HCC cells. Interestingly, CCL20 induced expression of vimentin and downregulated the expression of E-cadherin. These results extend the role of CCL20 in HCC to the context of chemokine-mediated EMT. Several chemokines have been shown to induce EMT in various tumors. Biswas *et al*[23] found that CXCL13-CXCR5 co-expression regulates EMT of breast cancer cells. Ploenes *et al*[24] reported that CCL18 induces EMT in lung cancer cells and elevates the invasive potential. Albert *et al*[25] found that CXCR4 induces EMT in patients with squamous cells carcinoma. Li *et al*[26] found that SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and EMT *in vitro* through non-canonical activation of hedgehog pathway. Matsushita *et al*[27] showed that CXCL16 plays an important role in liver metastasis of colorectal cancer through the induction of EMT. Our study provides another model of chemokine-mediated EMT, in which CCL20 may play a significant role in HCC progression and metastasis.

A variety of cell signaling pathways have been implicated in the process of EMT. Chang *et al*[28] found that EMT is associated with activation of the PI3K/AKT/mTOR pathway in prostate cancer radio-resistance. Tsai *et al*[29] reported that downregulation of PI3K/AKT and Wnt/β-catenin signaling cascades reverses EMT and inhibits breast cancer cell invasiveness. Choi *et al*[30] demonstrated that PI3K/AKT, ERK1/2 and Smad2/3 pathways are associated with transforming growth factor β1-induced EMT in human lung carcinoma cells. By examining protein expression in patient specimens and in HCC cells *in vitro* in our study, we showed that CCL20 expression was negatively associated with E-cadherin and positively associated with vimentin, p-AKT, and β-catenin expression. These results suggest that CCL20 expression is involved with the EMT process in HCC and there might be crosstalk between the PI3K/AKT and Wnt/β-catenin signaling pathways.

In conclusion, the present findings indicate that CCL20 expression is correlated with clinicopathologic factors, recurrence and survival in HCC patients, as well as the proliferation, migration and invasion of HCC cells. CCL20 induces EMT-like changes in HCC cells through activation of PI3K/AKT and Wnt/β-catenin signaling pathways. CCL20 may therefore represent a promising molecular marker for predicting outcomes and treatment of HCC patients undergoing resection surgery.

COMMENTS

Background

Chemokine ligand 20 (CCL20), also known as liver activation regulated chemokine or macrophage inflammatory protein-3, is a small cytokine that is strongly chemotactic for lymphocytes and weakly attracts neutrophils. Increasing evidence suggests that CCL20 is involved with tumor formation, progression and metastatic processes in many malignancies, including breast and colorectal cancer.

Research frontiers

Recent studies on CCL20 have mostly focused on the expression in hepatocellular carcinoma (HCC) tissues. Thus far, no studies have described the underlying mechanism by which CCL20 regulates the growth and metastasis of HCC cells. The results of this study provide new targets and a theoretical basis for the therapy of HCC.

Innovations and breakthroughs

This study demonstrates that CCL20 plays an important role in the proliferation and migration of HCC cells. CCL20 expression could induce an epithelial-mesenchymal transition (EMT)-like change in HCC cells *via* crosstalk between phosphoinositide kinase-3 (PI3K)/AKT and Wnt/β-catenin signaling pathways. CCL20 may be useful as a molecular monitor of metastasis and recurrence in HCC patients.

Applications

CCL20 induces EMT-like changes in HCC cells *via* crosstalk between PI3K/AKT and Wnt/β-catenin signaling pathways, which may therefore be used as indicators for anti-HCC therapy and prognosis evaluation.

Peer review

The authors detected expression of CCL20 in HCC tissues and analyzed its prognostic significance for HCC patients. They further identified the PI3K-AKT and Wnt pathways as possible mechanisms for the EMT-like phenotype induced by CCL20.

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ORIGINAL ARTICLE

Basic Study

Glucagon-like peptide-2 protects impaired intestinal mucosal barriers in obstructive jaundice rats

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Abstract

AIM: To observe the protective effect of glucagon-like peptide-2 (GLP-2) on the intestinal barrier of rats with obstructive jaundice and determine the possible mechanisms of action involved in the protective effect.

METHODS: Thirty-six Sprague-Dawley rats were randomly divided into a sham operation group, an obstructive jaundice group, and a GLP-2 group; each group consisted of 12 rats. The GLP-2 group was treated with GLP-2 after the day of surgery, whereas the other two groups were treated with the same concentration of normal saline. Alanine aminotransferase (ALT), total bilirubin, and endotoxin levels were recorded at 1, 3, 7, 10 and 14 d. Furthermore, on the 14th day, body weight, the wet weight of the small intestine, pathological changes of the small intestine and the immunoglobulin A (IgA) expressed by plasma cells located in the small intestinal lamina propria were recorded for each group.

RESULTS: In the rat model, jaundice was obvious, and the rats' activity decreased 4-6 d post bile duct ligation. Compared with the sham operation group, the obstructive jaundice group displayed increased yellow staining of abdominal visceral serosa, decreased small intestine wet weight, thinning of the intestinal muscle layer and villi, villous atrophy, uneven height, fusion, partial villous epithelial cell shedding, substantial inflammatory cell infiltration and significantly reduced IgA expression. However, no significant gross changes were noted between the GLP-2 and sham groups. With time, the levels of ALT, endotoxin and bilirubin in the GLP-2 group were significantly increased compared with the sham group ($P < 0.01$). The increasing levels of the aforementioned markers were more significant in the obstructive jaundice group than in the GLP-2 group ($P < 0.01$).

CONCLUSION: GLP-2 reduces intestinal mucosal injuries in obstructive jaundice rats, which might be attributed to increased intestinal IgA and reduced bilirubin and endotoxin.

Key words: Intestinal mucosal barrier; Glucagon-like

peptide-2; Obstructive jaundice

Core tip: It has recently been demonstrated that glucagon-like peptide-2 (GLP-2) has a highly tissue-specific trophic effect on the small intestine. However, whether GLP-2 also functions as an adapter for rats with obstructive jaundice is unknown. Studies on this topic are rare, and our research clearly illustrates that exogenous GLP-2 reduces intestinal mucosal injuries in an obstructive jaundice rat model. The next step of our study is to continue focusing on the details of this research as further studies are needed.

Chen J, Dong JT, Li XJ, Gu Y, Cheng ZJ, Cai YK. Glucagon-like peptide-2 protects impaired intestinal mucosal barriers in obstructive jaundice rats. *World J Gastroenterol* 2015; 21(2): 484-490 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/484.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.484

INTRODUCTION

Glucagon-like peptide-2 (GLP-2) is a 33-amino acid peptide encoded by the carboxy terminal of GLP-1 in proglucagon. It was first reported and introduced as a specific adapter of the intestinal mucosa by Drucker *et al*[1] in 1996. GLP-2 is co-secreted with GLP-1, oxyntomodulin and glicentin from enteroendocrine L cells, which are primarily located in the ileum and proximal colon. It has recently been demonstrated that GLP-2 has a highly tissue-specific trophic effect on the small intestine and augments the adaptive response to intestinal resection in the rat. Additionally, GLP-2 inhibits gastric acid secretion, enhances intestinal sugar transport and slows gastric emptying[2-5]. Halaçlar *et al*[6] reported that bacterial translocation in samples of the liver, spleen, mesenteric lymph nodes and portal and systemic blood obtained from the GLP-2 treated group was reduced compared with samples obtained from the colitis group. In addition, the Chinese scholars Li *et al*[7] discovered that the rate of bacterial translocation and the level of endotoxin in rats with gut ischemia-reperfusion injury were significantly increased compared with those treated by GLP-2. Above all, the most important property of GLP-2 in the gastrointestinal (GI) tract is its enterotrophic effect[1,8-10]. Therefore, its potential therapeutic role in patients with intestinal insufficiency secondary to extensive disease or resection of the small bowel is of interest[4,9].

Intestinal barrier function is damaged in patients with obstructive jaundice, potentially leading to bacteria translocation, endotoxemia and increased mortality within the peri-operative period. An increasing number of scientists and doctors have attempted to improve intestinal function in patients or rats with obstructive jaundice. Based on the aforementioned comments, we designed a study to observe whether GLP-2 acts on the damaged intestinal mucosa of rats with obstructive jaundice.

MATERIALS AND METHODS

Animals and experimental design

Male Sprague-Dawley rats, weighing 200-250 g, were housed under controlled temperature, humidity and 12-h dark/light cycles; the rats were housed in stainless-steel cages and provided free access to water and rat chow before and after the operation. The rats were randomized into three groups (n = 12 in each group). Group 1 (Control; C) underwent sham operation, whereas Group 2 [obstructive jaundice (OB)] underwent common bile duct ligation. Both of the groups were given simultaneous treatment with the same amount of normal saline after the surgeries. Group 3 [obstructive jaundice with GLP-2 (OBGLP-2)] underwent common bile duct ligation and simultaneous treatment with GLP-2 [0.2 μg/(g•d)]. Either normal saline or GLP-2 was administered by peritoneal injection daily.

Operative procedures

Using sterile techniques, a midline incision was created. The common bile duct was identified, double ligated with 5-0 silk and divided between the two ligatures. In sham-operated animals, the common bile duct was freed from the surrounding soft tissue without ligation and transection. The operation was performed using 100 g/L chloral hydrate (3 mL/kg) for intraperitoneal anesthesia.

Blood biochemistry

Serum samples were obtained and analyzed on postoperative days 1, 3, 7, 10 and 14. All serum samples were measured using a Hitachi 7600 modular chemistry analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) to determine the levels of alanine aminotransferase (ALT) and total bilirubin (Tbil) using kits from Jiancheng Bioengineering Institute. The endotoxin levels in plasma were measured according to the manufacturer's instructions with the kits (Horseshoe Crab Reagent, Xiamen, China).

Tissue harvest and histopathological evaluation

After 2 wk, the animals were anesthetized, and repeat laparotomy was performed. Segments of the small bowel, approximately 3 cm long, were harvested from the terminal ileum, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) and immediately snap-frozen in liquid nitrogen for immunohistochemistry. Then, the samples were fixed in 400 g/L of paraformaldehyde solution. Sections of 5 μm were cut and stained with hematoxylin and eosin (HE). The pathological changes and injuries to the intestinal mucosa were evaluated under a light microscope by an independent observer who was blinded to the experimental protocol. Additionally, secretory

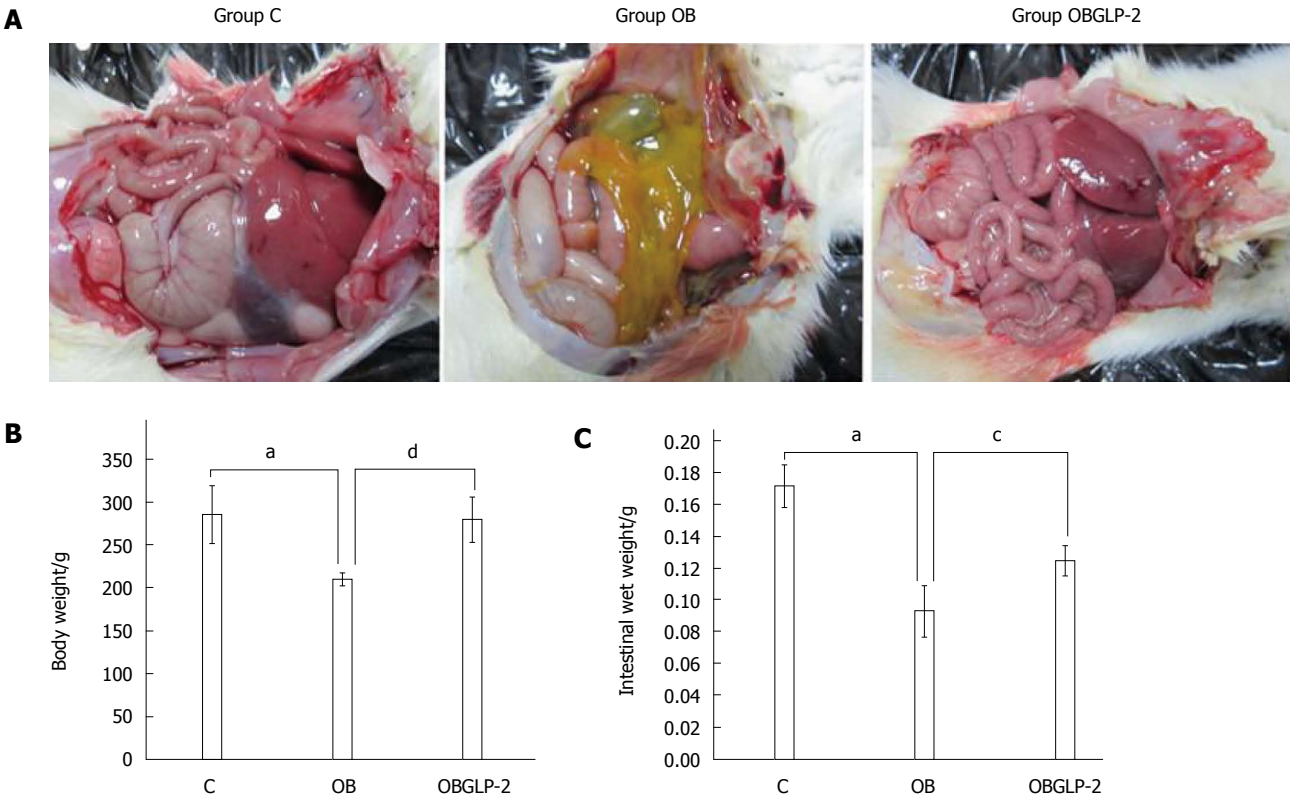

Figure 1 Glucagon-like peptide-2 protects impaired intestinal mucosa on day 14. A: Mesentery anatomy of the three groups; B: Body weights of the rats in three groups on day 14; C: Wet intestinal weights of the rats in the three groups on day 14. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$, $^{d}P < 0.01$, OB *vs* OBGLP-2;. C: Control; OB: Obstructive jaundice; OBGLP-2: Obstructive jaundice with GLP-2.

immunoglobulin A (sIgA) expression in the tissue samples was assessed by immunohistochemical analysis.

Statistical analysis

Experimental data were analyzed with the SPSS 13.0 statistical program (statistical product and service solutions, © 1999 to 2003; SPSS Institute Inc., Armonk, NY, United States). The results are expressed as mean ± SD. The differences among groups were evaluated by the homogeneity test of variance analysis. The means of independent samples were analyzed and compared with the independent sample *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Rat observations

Two to three days after bile duct ligation, the ears and tails started to exhibit jaundice, and the urine turned yellow. After 4-6 d, the jaundice was obvious, the stools were pale, and the activity of the rats decreased. Four rats (1 in group C, 1 in group OBGLP-2 and 2 in group OB) died of complications, including abdominal infection, malnutrition, liver function failure and water and electrolyte disorders. The remaining rats survived until they were sacrificed at the end of the experiments.

Gross mesentery observation

Unlike the sham group, the organ serosa of the jaundiced rats exhibited yellow discoloration, and the root mesenteric lymph nodes displayed beadlike enlargement. However, on the 14th day, the GLP-2 group exhibited no parietal peritoneum changes (Figure 1A). The mean body weight and wet intestinal weight were reduced in the jaundiced group compared with the sham group (Figure 1B and C); however, GLP-2 reduced the reduction in both body weight and wet intestinal weight, indicating that GLP-2 protects the intestinal mucosa from damage.

Small intestinal pathology and alterations in sIgA expression

The sham group exhibited normal villous formation in the intestine tissue with equal height (Figure 2A). However, the jaundiced group displayed intestinal muscularis layer thinning with villous thinning, atrophy, uneven height, villous fusion, partial villous epithelium shedding and considerable lymphocyte infiltration (Figure 2B). The GLP-2 group displayed villi that were more equal in height compared with the jaundiced group as well as slight villous edema with no epithelial shedding (Figure 2C). In the sham group, the intestinal crypts exhibited minimal sIgA-stained cells (Figure 2D). The jaundiced group had shallow and disorganized intestinal

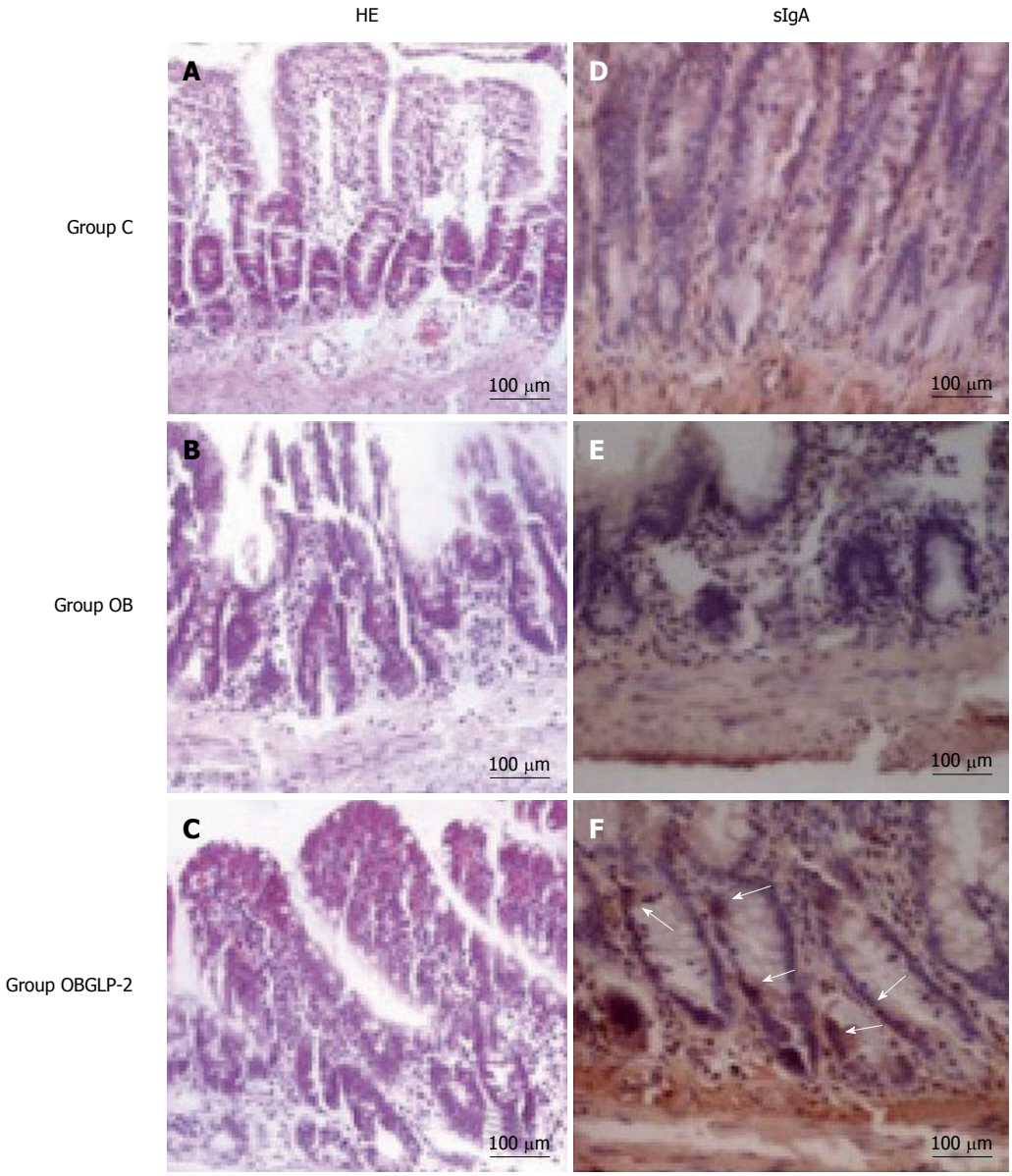

Figure 2 Intestinal mucosal pathological alterations as assessed by HE staining and sIgA immunohistochemical appearance on day 14. C: Control; OB: Obstructive jaundice; OBGLP-2: Obstructive jaundice with GLP-2.

crypts with almost no IgA-stained cells (Figure 2E). The GLP-2 group exhibited deeper and more organized intestinal tissue crypts than the jaundiced group with localized positive sIgA staining (Figure 2F).

Alterations in serum Tbil, ALT and endotoxin levels

The jaundiced and GLP-2 groups exhibited obvious ALT elevations compared with the sham group. However on postoperative days 3, 7, 10, and 14, the GLP-2 group had reduced ALT levels compared with the jaundiced group (Figure 3A, $P < 0.05$). The jaundiced and GLP-2 groups showed obvious elevations in endotoxin levels compared with the sham group, and the level continued to rise as time passed. However, on days 10 and 14, the endotoxin level in the GLP-2 group was significantly reduced compared with the jaundiced group on the same days (Figure 3B, $P < 0.05$). The jaundiced and GLP-2 groups exhibited an obvious increase in the serum bilirubin compared with the sham group. However, on postoperative days 10 and 14, the GLP-2 group exhibited a significantly lower level of serum bilirubin compared with the jaundiced group (Figure 3C, $P < 0.05$).

DISCUSSION

Obstructive jaundice can cause a host of complex and severe pathological and physiological changes to various organs. Intestinal mucosal barrier dysfunction and intestinal immune function decline might result in endotoxemia and intestinal bacterial translocation, which are important contributors to disease progression or death in patients[11-13]. Under normal circumstances, the large number of bacteria in the intestinal tract cannot enter the body tissue or blood circulation due to the mechanical,

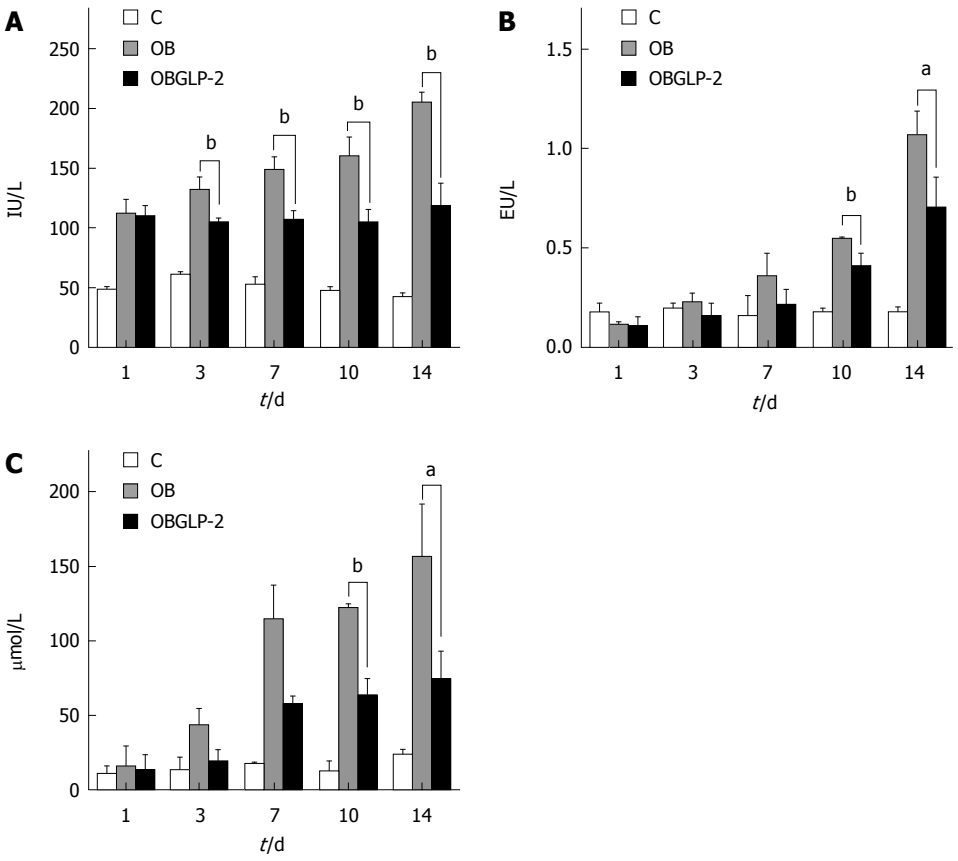

Figure 3 Comparison of serum alanine aminotransferase (A), total bilirubin (B) and endotoxin levels (C) in three groups (n = 12). [a]P < 0.05, [b]P < 0.01, OB *vs* OBGLP-2. C: Control; OB: Obstructive jaundice; OBGLP-2: Obstructive jaundice with GLP-2.

biological, chemical and immunological barriers of the intestine. When the intestinal mechanical barrier is damaged, endotoxins enter the blood circulation, causing endotoxemia. Then, the intestinal bacteria can migrate into the intestinal lymph nodes, blood, liver or spleen, causing bacterial translocation[14-20].

In this study, we observed that rats with obstructive jaundice exhibit circular muscularis thinning and decreased mucosal thickness and villous height. Obstructive jaundice is associated with intestine mucosal structural changes and a decline in protein synthesis in the liver. These effects are coupled with a lack of proteins for gastrointestinal epithelium regeneration and renewal, leading to intestinal mechanical barrier damage and bacterial translocation. GLP-2 is an intestinal epithelial specific growth factor, and its main role is to stimulate intestinal crypt cell proliferation and inhibit cell apoptosis, promoting the growth of the intestinal mucosa and regeneration after injury[18]. GLP-2 also inhibits gastric acid secretion and gastric motility, increases the intestinal blood supply, improves intestinal barrier function and promotes the intestinal absorption of nutrients[19,20]. However, studies regarding the use of rhGLP-2 in obstructive jaundice to improve the intestinal barrier function and immune function are rare. As seen from the 14-d results of this experiment, rats receiving subcutaneous exogenous GLP-2 exhibited less structural damage to the intestinal mucosa and tall intestinal villi that were neat and relatively intact compared with the jaundiced group. This indicates that exogenously administered GLP-2 aids in the protection and improvement of the intestinal mechanical barrier in rats with obstructive jaundice.

Additionally, when obstructive jaundice occurs, the impaired local intestinal immune function is also one of the factors responsible for bacterial translocation. The local intestinal mucosal immune system primarily consists of intestinal lymphocytes, lymphoid tissue, plasma cells and immunoglobulins[21,22]. Among the immunoglobulins, sIgA plays an important role. sIgA neutralizes viruses, toxins and the biological activity of antigen enzymes and prevents bacterial adhesion on the surface of intestinal epithelial cells. sIgA displays a synergistic bactericidal effect with the complement system and lysozymes. Therefore, sIgA is an important factor facilitating the protection of the intestinal barrier function that prevents bacterial translocation[23]. In this study, ileal biopsy immuno-histochemistry indicated that rats with obstructive jaundice display significantly decreased expression of ileal sIgA compared with the control group. However, the GLP-2 group exhibited significantly increased IgA expression compared with the obstructive jaundice group. These results indicate that by repairing damage to the integrity of intestinal epithelial cells and increasing sIgA synthesis in ileal epithelial cells, GLP-2 can protect rats with obstructive jaundice from intestinal barrier dysfunction and immune system damage. As an

intestinal epithelium-specific growth factor, GLP-2 has a strong effect on the recovery of intestinal epithelial injury, and it is stronger than any other non-specific intestinal epithelial growth factor[24-27]. These advantages of GLP-2 suggest that it might have useful clinical applications.

With respect to its mechanism of action on the intestinal mucosa, a large number of studies have demonstrated that GLP-2 exerts its actions *via* a G protein-coupled receptor (GLP-2R). The human *GLP-2R* gene is located on chromosome 17p13.3[28,29]. GLP-2R is a member of the G protein-coupled receptor superfamily and has 7 transmembrane domains. GLP-2R and glucagon as well as GLP-1 and the glucose-dependent insulinotropic polypeptide receptor are highly homologous. GLP-2R is widely distributed in intestinal epithelial cells, gastric epithelial cells, enteric neurons, intestinal endocrine cells[29], intestinal submucosal myofibroblasts, islet A cells, the brain and lungs[30,31]. New research findings demonstrate that GLP-2 promotes the growth of normal small bowel and the recovery of pathologic intestinal mucosa through multiple pathways. On one hand, GLP-2 promotes proliferation of the intestinal mucosa by binding to GLP-2R, which is distributed in intestinal epithelial cells. On the other hand, GLP-2 protects the intestinal function indirectly by binding to the GLP-2R, which is distributed in other regions. The effects of GLP-2 involve many cell signal transduction pathways, mainly the cAMP/PKA, PI3K/Akt and Wnt/β-catenin pathways, of which the cAMP/PKA pathway is the main one. These pathways coordinate and regulate intestinal epithelial cells, promoting steady development and intestinal adaptation. However, the mechanism(s) by which these pathways coordinate and integrate key control points and feedback inhibition are not entirely clear, and further studies are required[32-35].

Above all, GLP-2 has widely been accepted as an adapter of the intestinal mucosa. In this study, we also observed its protective function in an obstructive jaundice rat model. This effect might be attributed to increased intestinal IgA and reduced bilirubin and endotoxin. In December 2012, the United States Food and Drug Administration approved the use of Teduglutide, a GLP-2 analog, to treat adults with short bowel syndrome who need additional nutrition from intravenous feeding (parenteral nutrition). Will the drug's application expand if more experiments clarify the protection of the intestinal mucosa in rats or humans with obstructive jaundice?

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COMMENTS

Background

Obstructive jaundice can cause a host of complex and severe pathological injuries to the intestinal mucosal barrier and result in intestinal immune function decline, endotoxemia and intestinal bacterial translocation, which are important factors in disease progression or death in patients. Many recent studies have demonstrated that glucagon-like peptide-2 (GLP-2) can help protect and improve intestinal mechanical barrier function in rats. However, few direct studies on whether GLP-2 has a protective role in obstructive jaundice animal models are available.

Research frontiers

Many researchers have discussed the mechanism by which GLP-2 acts on the intestinal mucosa and signal pathways. Many scientists are attempting to determine whether GLP-2 is related to neoplastic development. In addition, the new GLP-2 analog Teduglutide was approved by the United States Food and Drug Administration to treat adults with short bowel syndrome. Unfortunately, to date, few studies have discussed the relationship between GLP-2 and injuries caused by obstructive jaundice.

Innovations and breakthroughs

Recent reports have highlighted the function of GLP-2 as an adapter of intestinal mucosa. Almost no direct studies evaluating whether GLP-2 has a protective function on obstructive jaundice rats were identified from a PubMed search. This study is the first to report that GLP-2 efficiently prevents intestinal mucosa injury after bile duct ligation operations in a rat model. These effects might be attributed to an increase in intestinal IgA, reduced bilirubin and endotoxin and improved liver function.

Applications

Due to its specific protective function in the intestinal mucosa, GLP-2 can be widely used in patients with intestinal barrier injury despite the cause.

Terminology

GLP-2 is a pleiotropic hormone that affects multiple facets of intestinal physiology, including growth, barrier function, digestion, absorption, motility and blood flow. The mechanisms through which GLP-2 produces these actions are complex, involving unique signaling mechanisms and multiple indirect mediators. However, few studies have investigated its function in rats or humans with obstructive jaundice. This study was designed to fill this gap in knowledge.

Peer review

This paper demonstrates the usefulness of GLP-2 in rats with obstructive jaundice. The study is well designed, and the manuscript is interesting and clearly described.

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ORIGINAL ARTICLE

Basic Study

E2F-1 overexpression inhibits human gastric cancer MGC-803 cell growth *in vivo*

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Abstract

AIM: To evaluate the influence of E2F-1 on the growth of human gastric cancer (GC) cells *in vivo* and the mechanism involved.

METHODS: E2F-1 recombinant lentiviral vectors were injected into xenograft tumors of MGC-803 cells in nude mice, and then tumor growth was investigated. Overexpression of transcription factor E2F-1 was assessed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analysis. Apoptosis rates were determined using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Expression levels of certain cell cycle regulators and apoptosis-related proteins, such as Bax, survivin, Bcl-2, cyclin D1, S-phase kinase-associated protein 2, and c-Myc were examined by Western blotting and RT-PCR.

RESULTS: Xenograft tumors of MGC-803 cells in nude mice injected with E2F-1 recombinant lentiviral vectors stably overexpressed the *E2F-1* gene as measured by semi-quantitative RT-PCR (relative mRNA expression: 0.10 ± 0.02 *vs* 0.05 ± 0.02 for control vector and 0.06 ± 0.03 for no infection; both $P < 0.01$) and Western blotting (relative protein expression: 1.90 ± 0.05 *vs* 1.10 ± 0.03 in control vector infected and 1.11 ± 0.02 for no infection; both $P < 0.01$). The growth-curve of tumor volumes revealed that infection with E2F-1 recombinant lentiviral vectors significantly inhibited the growth of human GC xenografts (2.81 ± 1.02 *vs* 6.18 ± 1.15 in control vector infected and 5.87 ± 1.23 with no infection; both $P < 0.05$) at 15 d after treatment. TUNEL analysis demonstrated that E2F-1 overexpression promoted tumor cell apoptosis (18.6% ± 2.3% *vs* 6.7% ± 1.2% in control vector infected 6.3% ± 1.2% for no infection; both $P < 0.05$). Furthermore, lentiviral vector-mediated E2F-1 overexpression increased the

expression of Bax and suppressed survivin, Bcl-2, cyclin D1, Skp2, and c-Myc expression in tumor tissue.

CONCLUSION: E2F-1 inhibits growth of GC cells *via* regulating multiple signaling pathways, and may play an important role in targeted therapy for GC.

Key words: E2F-1; Gastric cancer; Lentiviral vector; Mouse model

Core tip: Transcription factor E2F-1 is the prototypical E2F and is often implicated in DNA synthesis and repair, cell proliferation, and apoptosis. Our preliminary study revealed that high expression of E2F-1 significantly suppressed gastric cancer (GC) cell line progression *in vitro*. However, the role of E2F-1 overexpression in GC *in vivo* remains unknown. Our results showed that overexpression of E2F-1 significantly inhibited tumor growth and promoted tumor cell apoptosis *in vivo*. Survivin, Bcl-2, cyclin D1, S-phase kinase-associated protein 2 and c-Myc were upregulated, and Bax was downregulated by E2F-1. E2F-1 inhibits growth of GC cells *via* regulating multiple signaling pathways.

Wei WY, Yan LH, Wang XT, Li L, Cao WL, Zhang XS, Zhan ZX, Yu H, Xie YB, Xiao Q. E2F-1 overexpression inhibits human gastric cancer MGC-803 cell growth *in vivo*. *World J Gastroenterol* 2015; 21(2): 491-501 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/491.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.491

INTRODUCTION

Although the incidence rate of gastric cancer (GC) has gradually decreased recently, it remains the second leading cause of cancer-related death worldwide[1]. Of all GC cases, > 70% occur in developing countries and half of the total cases worldwide occur in Eastern Asia (mainly China)[2]. Despite improvements in surgical techniques and the development of new chemotherapeutic regimens, patient outcome is often disappointing. Patients are mostly diagnosed at advanced stages, typically with a poor prognosis, with a five-year survival rate of < 30%[3]. Because of the patient's own reasons, they lose the opportunity to receive surgery and chemotherapy[4]. Thus, it is necessary to find new therapies. Gene therapy as a novel strategy has been shown to have a therapeutic advantage for treating several types of tumors, including gastric carcinoma, and promises to be a new therapeutic approach to inhibit the proliferation of tumor cells, and avoids the side effects of drug therapy[5,6].

Since the E2F family factors have been reported, they have been considered as main regulators of cell growth and proliferation[7]. *E2F-1* gene is one member of the E2F family, with the ability to induce apoptosis independently[8]. E2F-1 is also a key regulator for the G1/S phase transition[9]. On the one hand, a number of researchers have shown that high expression of E2F-1 is a risk factor for malignant tumors[10,11]. On the other hand, E2F-1 overexpression may play an important role in suppressing tumor growth in lung cancer, breast cancer and osteosarcoma[12-14]. These findings indicate that the *E2F-1* gene has a dual effect in promoting cell proliferation and apoptosis. However, few studies have been reported concerning E2F-1 expression in GC. In particular, the functional mechanism of E2F-1 overexpression has not been determined. Our previous study indicated that E2F-1 overexpression had a significant influence on cell cycle progression and proliferation in an *in vitro* GC cell model[15,16], but the molecular mechanisms underlying inhibition of cell growth and increase of apoptosis by E2F-1 overexpression remain unclear.

It is widely known that undifferentiated cells and differentiated cells can be efficiently infected with lentivirus, and lentivirus-carrying genes are stably integrated into the host genome[17,18]. Therefore, transfer of recombinant lentiviral vectors is the best transgene method in various animals. Accordingly, we constructed E2F-1 recombinant lentiviral vectors and evaluated the influence of E2F-1 overexpression on the biologic behavior of MGC-803 cells *in vivo* using a xenograft tumor model. To explore the potential mechanism, we also examined the influence of E2F-1 overexpression on the expression of survivin, Bax, Bcl-2, cyclin D1, S-phase kinase-associated protein (Skp)2, and c-Myc in MGC-803 cells *in vivo*.

MATERIALS AND METHODS

Cell culture

The human GC MGC-803 cells and human kidney 293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were incubated in Dulbecco's modified eagle medium (HyClone of Thermo Fisher Scientific Inc., Waltham, MA, United States), supplemented with 2 mmol/L glutamine, 0.05 g/L penicillin, 0.1 g/L streptomycin, and 10% fetal bovine serum at 37 ℃ in a humidified atmosphere containing 5% CO_2. Culture media were replaced once every two days.

Antibodies

Specific rabbit anti-human antibodies to E2F-1, c-Myc, Skp2, Bax, Bcl-2, cyclin D1, survivin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Abcam (Cambridge, United Kingdom). Infrared-labeled secondary goat anti-rabbit antibodies to IRDye 800 were obtained from Li-Cor Biosciences (Lincoln, NE, United States).

Construction of E2F-1 recombinant lentiviral vectors

Lentiviral vectors with green fluorescent protein (GFP) were provided by Genechem (Shanghai, China). The

Table 1 Sequences of primers used for semi-quantitative real-time polymerase chain reaction

Gene	Primer	Base sequence	PCR product (bp)
E2F-1	Forward	5'-CCCAACTCCCTCTACCCT-3'	217
	Reverse	5'-CTCCCATCTCATATCCATCCTG-3'	
Survivin	Forward	5'-AAATGCACTCCAGCCTCTGT-3'	311
	Reverse	5'-TGTCGAGGAAGCTTTCAGGT-3'	
Bax	Forward	5'-CCAAGAAGCTGAGCGAGTGT-3'	269
	Reverse	5'-CCGGAGGAAGTCCAATGTC-3'	
Bcl-2	Forward	5'-GACTTCGCCGAGATGTCCAG-3'	259
	Reverse	5'-CATCCCAGCCTCCGTTATCC-3'	
Cyclin D1	Forward	5'-CCCTCGGTGTCCTACTTCAA-3'	237
	Reverse	5'-GGGGATGGTCTCCTTCATCT-3'	
Skp2	Forward	5'-GCTGCTAAAGGTCTCTGGTGT-3'	291
	Reverse	5'-AGGCTTAGATTCTGCAACTTG-3'	
c-Myc	Forward	5'-TTCTCTCCGTCCTCGGATTC-3'	282
	Reverse	5'-GTAGTTGTGCTGATGTGTGG-3'	
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'	450
	Reverse	5'-TCACCACCCTGTTGCTGTA-3'	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PCR: Polymerase chain reaction; Skp2: S-phase kinase-associated protein 2.

lentiviral vector system was comprised of the vectors pGCL-GFP, pHelper 1.0 and pHelper 2.0. The full-length human *E2F-1* gene (NM_0005225.2) was encoded by the pGCL-GFP-E2F-1 plasmid. The E2F-1 cDNA was accurately inserted into the plasmid, which was verified by DNA sequencing and PCR technology. The three plasmids (pHelper 1.0, pHelper 2.0, and pGCL-GFP or pGCL-GFP-E2F-1) were co-transfected into 293T cells using Lipofectamine 2000[19]. After a 12-h transfection, the medium was replaced with fresh medium supplemented with 10% fetal bovine serum. The lentivirus containing the E2F-1 gene was collected at 48 h after the transfection. The product was concentrated by ultracentrifugation (12000× *g*) and diluted in a series of gradients from 10^{-2} to 10^{-5}. Various dilutions of lentivirus were used to transfect 293T cells. Then the cells were observed under a fluorescence microscope after 72 h. The cells with GFP expression were positive cells, and the number of positive cells was counted in each transfection. The lentiviral titer was calculated by the formula: lentiviral titer (TU/mL) = number of positive cells × dilution times/volume of lentivirus used.

Xenograft tumor model

Athymic nude male BALBC/c mice, aged 4-5 wk, were purchased from Guangxi Animal Center (Nanning, China). The mice were maintained in specific pathogen-free, temperature-controlled isolation conditions and fed with sterilized food and autoclaved water according to the experimental animal guidelines. All animal procedures were conducted to follow the provisions of the Ethics Committee of Guangxi Medical University in the research. Xenografted tumor models were prepared by subcutaneous injection of 3×10^6 MGC-803 cells suspended in phosphate-buffered saline (PBS) into male athymic nude mice. The xenografted tumor was referred to as a human GC xenograft.

Treatment of human GC xenografts in nude mice

When tumors reached a diameter of approximately 6 mm, mice were randomized into three groups (n = 8 each): LV-GFP-E2F-1, LV-GFP-NC, and PBS. The tumors of nude mice were injected with LV-GFP-E2F-1 or LV-GFP-NC at a concentration of 5×10^6 TU in 200 μL PBS, while the control group received an equal volume of PBS. The tumors of animals were injected in each group once every 2 d. Animals and tumors were observed daily. Tumor size was monitored every 2 d with a digital caliper and tumor volume (TV) was estimated by the equation: $TV = a \times b^2/2$, where a is the longest diameter and b the shortest diameter. The relative tumor volume (RTV) was calculated by the formula: $RTV = V_t/V_0$ (V_0 was the initial TV of the first day of treatment and V_t was the following TV measured)[20]. After a total of 15 d of treatment, nude mice were suffocated and tumors were assessed.

Semi-quantitative reverse transcriptase-PCR

Total RNA was extracted from MGC-803 tumor tissues and GC xenografts with TRI Reagent (Sigma-Aldrich, St Louis, MO, United States). cDNA was generated from DNase-1-treated RNA template with 0.2 μg random hexamer primers and 200 U RevertAid H-Minus M-MuLV reverse transcriptase enzyme (Roche, Switzerland). All the PCR primer sequences used in this study, including for c-Myc, Skp2, Bax, Bcl-2, cyclin D1, surviving and GAPDH are shown in Table 1. The PCR products were used for electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.5 μg/mL). The mRNA expression levels of each gene were measured using *GAPDH* mRNA as a reference. Reverse transcriptase (RT)-PCR product bands were analyzed by

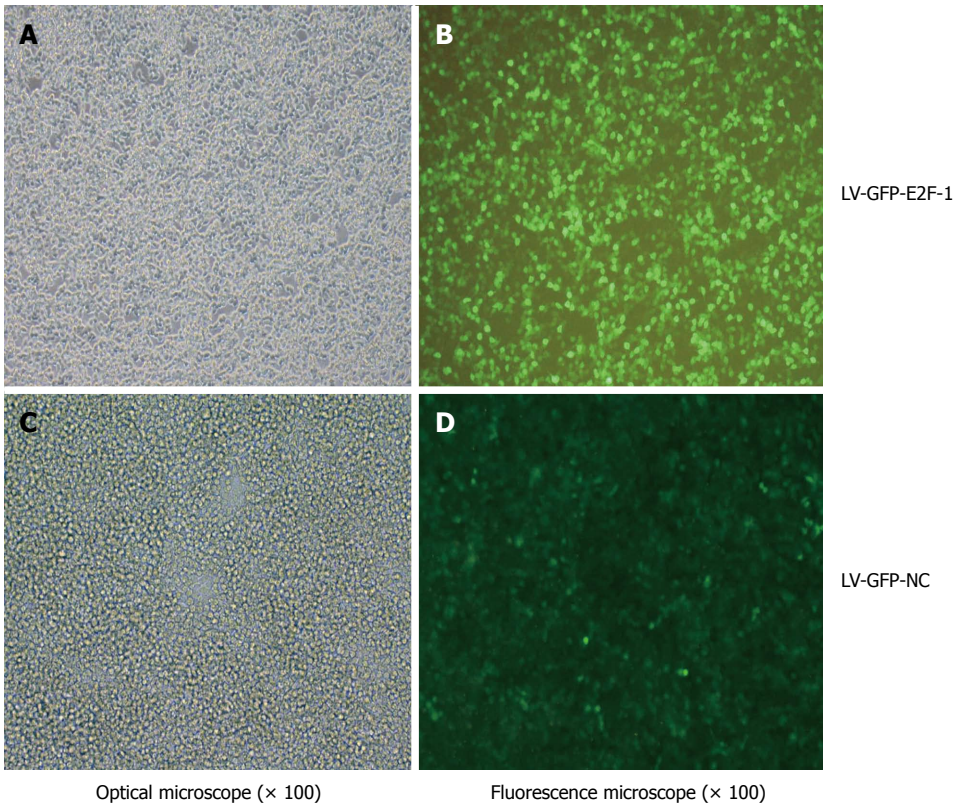


Figure 1 Determination of lentiviral titers. Recombinant lentivirus was transfected into 293T cells to determine viral titer by the method of end-point dilution, which involves counting the numbers of infected green cells under fluorescence microscopy (original magnification x 100). The viral dilution factor was 1:1000.

densitometry using Quantity One 1-D gel image analysis software (BioRad, Hercules, CA, United States).

Western blot analysis

Protein was extracted in accordance with the kit instructions (OriGene, Rockville, MD, United States) in tumor tissue. Proteins were separated by 10% polyacrylamide-gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Roche) at 100 mA for 3 h, and later soaked for 2 h in a blocking solution (Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20), and immersed in a 1:1000 anti-E2F-1 antibody diluent, or anti-GAPDH monoclonal antibody used as an internal control, then incubated at 4 ℃ overnight. The membrane was washed with PBS with Tween 20 and immersed in a 1:10000 infrared-labeled secondary goat anti-rabbit antibody diluent for 30 min. The membranes were analyzed by densitometry using Odyssey version 3.0 (Li-Cor). Then the protein expression levels of each gene were measured using GAPDH protein as a reference.

Analyses of GC xenograft tissue apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

Paraffin sections of GC xenografts were processed with a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (Roche) treated according to the manufacturer's instructions. Gastric tumor cell apoptosis was observed under microscopy. The primary antibody was replaced by phosphate buffer on the positive specimens in the negative controls. The brown particles in the nucleus were considered as apoptosis-positive cells. Eight fields were randomly selected in each slice under a high-power field (×400). The percentage of TUNEL-positive cells was calculated from a total of 200 cells in each field.

Statistical analysis

Data analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, United States). Numerical data are reported as the mean ± SD. Group comparisons were evaluated by one-way analysis of variance. Two-sided tests were used to evaluate comparisons. Data were determined to have significant differences when $P < 0.05$.

RESULTS

Evaluation of pGCL-GFP-E2F-1 lentiviral vector

Positive clones were screened by restriction enzyme analysis, and were verified by DNA sequencing (data not displayed). Positive clone sequencing was consistent with the target sequence of E2F-1 reported in Genebank indicating that the pGCL-GFP-E2F-1 had been constructed successfully.

Determination of lentiviral titers

As displayed in Figure 1, lentiviral vectors of LV-GFP-

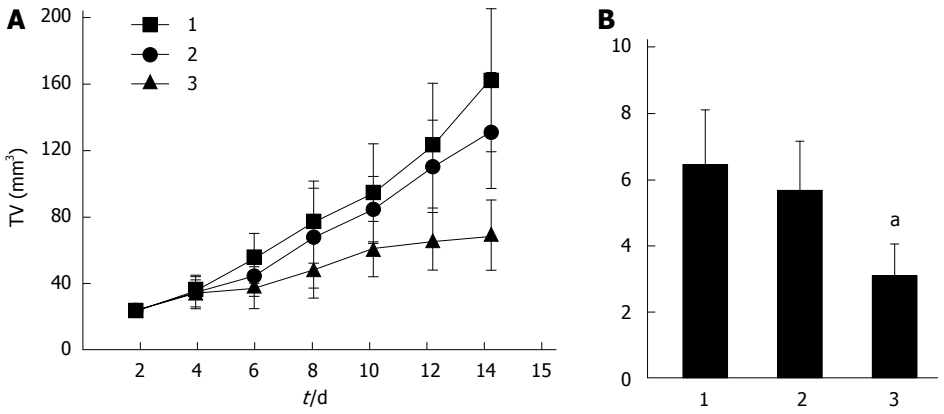


Figure 2 Overexpression of E2F-1 inhibits MGC-803 tumor growth. A: Tumor growth curve shows the growth tendency in the LV-GFP-E2F-1 group is suppressed in comparison to the control groups; B: Relative tumor volume (TV) in LV-GFP-E2F-1-treated mice was significantly smaller than in the control groups at 12 d after tumor injection (n = 8 animals for each condition). 1, phosphate-buffered saline (PBS) control group; 2, LV-GFP-NC group; 3, LV-GFP-E2F-1 group; comparisons made using analysis of variance and Student-Newman-Keuls analyses; $^aP < 0.05$ *vs* control.

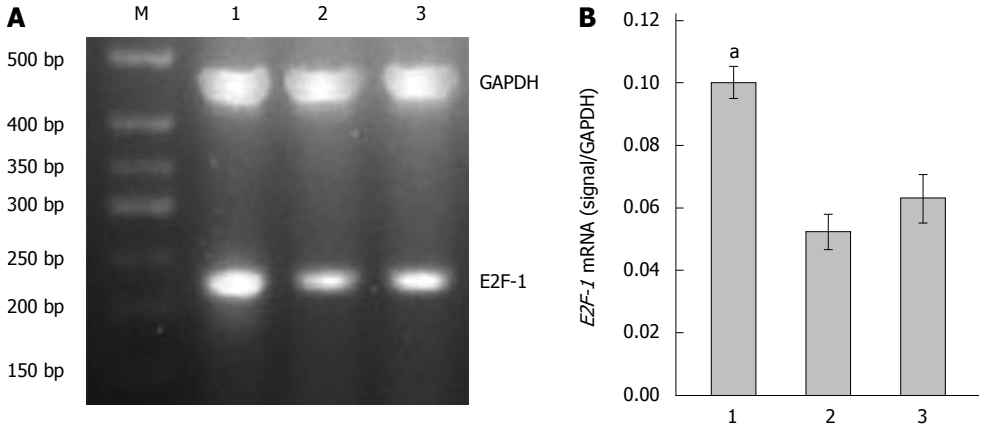


Figure 3 Overexpression of E2F-1 mRNA with LV-GFP-E2F-1. A: Agarose gel analysis of E2F-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) real-time polymerase chain reaction products amplified from MGC-803 tumor tissues (M, 500 bp marker); B: Expression of E2F-1 mRNA was measured in the three groups and normalized to GAPDH (n = 8 animals for each condition). 1, LV-GFP-E2F-1 group; 2, LV-GFP-NC group; 3, phosphate buffered saline control group. $^aP < 0.05$ using analysis of variance and Student-Newman-Keuls analyses.

E2F-1 or LV-GFP-NC were mixed into 293T cell culture medium, and more than 90% of the cells were transfected. The concentration of the virus was greater than 6×10^8 TU/mL by GFP expression assay.

Overexpression of E2F-1 inhibited GC xenograft tumor growth

The tumors of the GC xenograft model were injected with E2F-1 recombinant lentiviral vectors. Compared with the LV-GFP-NC and PBS groups, the growth-curve of tumor volumes (Figure 2A) revealed that the growth of xenografts infected with E2F-1 recombinant lentiviral vectors had was significantly inhibited ($P < 0.05$). After a total of 15 d of treatment, GC xenograft tumor volume in the LV-GFP-E2F-1 group was significantly smaller than in the LV-GFP-NC and PBS groups (both $P < 0.05$), and there was no statistically significant difference between LV-GFP-NC and PBS groups (Figure 2B). The results indicated that overexpression of E2F-1 could effectively suppress gastric cancer xenograft tumor growth *in vivo*.

Protein and mRNA of E2F-1 gene were highly expressed in GC xenograft tissue

Compared with the LV-GFP-NC and PBS groups, mRNA and protein expressions of E2F-1 gene were significantly higher in the LV-E2F-1-GFP group by gray value analysis ($P < 0.05$), and there was no statistically significant difference between LV-GFP-NC and PBS groups (Figures 3 and 4). The results suggest that a nude mouse model with overexpression of *E2F-1* was constructed successfully by injecting with E2F-1 recombinant lentiviral vectors.

Overexpression of E2F-1 induced GC xenograft tissue cell apoptosis

Cell apoptosis was detected by the TUNEL staining in the GC xenograft tissue (Figure 5). The rate of cell apoptosis in the LV-GFP-NC, LV-GFP-NC and PBS groups were 18.6% ± 2.3%, 6.7% ± 1.2% and 6.3% ± 1.2%, respectively. Compared with LV-GFP-NC and PBS groups, the apoptosis rate of the LV-E2F-1-GFP group

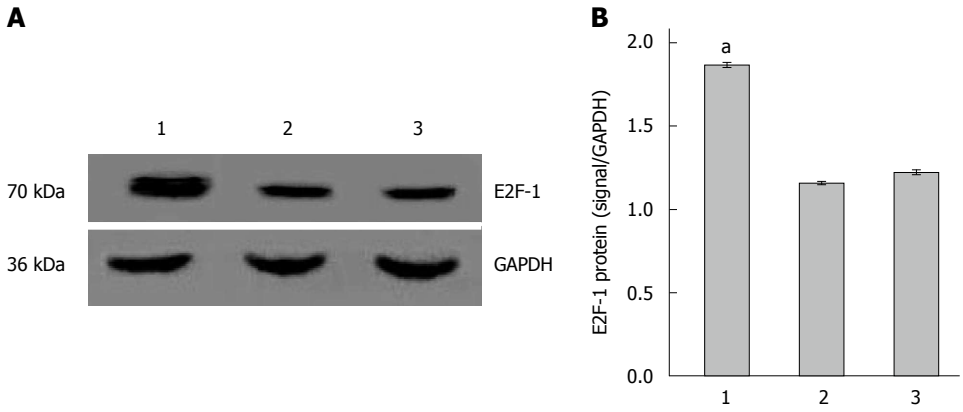


Figure 4 Overexpression of E2F-1 protein with LV-GFP-E2F-1. A: Western blot analysis of E2F-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; internal control) in MGC-803 tumor tissue; B: Expression of E2F-1 protein was measured in the three groups normalized to GAPDH (n = 8 animals for each condition). 1, LV-GFP-E2F-1 group; 2, LV-GFP-NC group; 3, PBS group. [a]$P < 0.05$ using analysis of variance and Student-Newman-Keuls analyses.

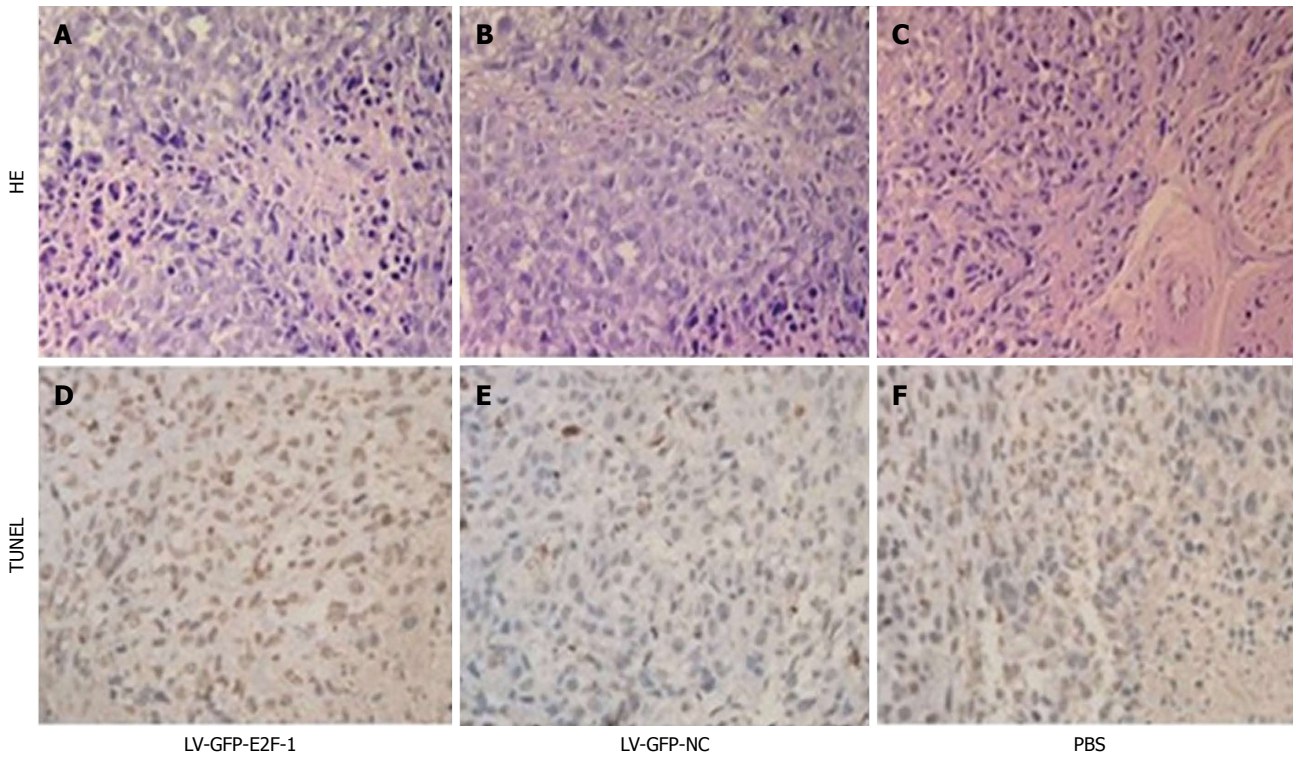
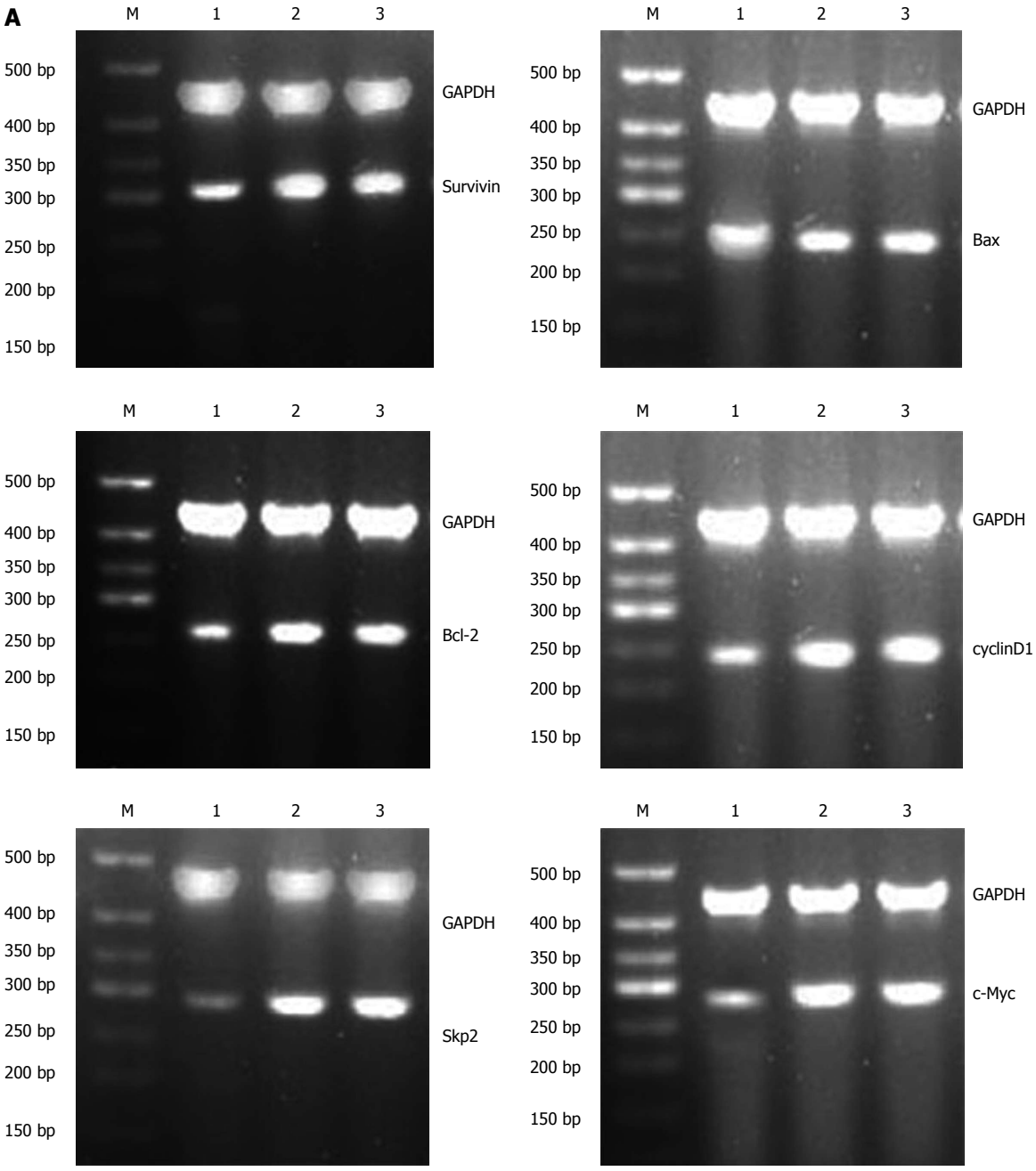


Figure 5 Overexpression of E2F-1 induces *in situ* MGC-803 tumor cell apoptosis. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and hematoxylin and eosin staining. More apoptotic MGC-803 tumor cells were observed in the LV-GFP-E2F-1 group than in the LV-GFP-NC and phosphate buffered saline (PBS) groups (original magnification, × 400).

was significantly higher ($P < 0.05$). The results suggest that overexpression of E2F-1 effectively promotes GC xenograft tumor cell apoptosis *in vivo*.

Overexpression of E2F-1 decreases expression of c-Myc, Skp2, Bcl-2, cyclin D1and survivin, and increases Bax expression

To probe the principles of E2F-1-induced cell apoptosis in GC xenografts, semiquantitative RT-PCR and Western blot were used to detect expression of apoptosis-related genes. Compared with the LV-GFP-NC and PBS groups, densitometry displayed that the mRNA levels of *c-Myc*, *Skp2*, *Bcl-2*, cyclin D1 and survivin were lower in the LV-GFP-E2F-1 group, whereas *Bax* levels were higher in the LV-GFP-E2F-1 group (all *P*s < 0.05), and there was no statistically significant difference between LV-GFP-NC and PBS groups (Figure 6). Compared with LV-GFP-NC and PBS groups, densitometry showed that the protein levels of c-Myc, Skp2, Bcl-2, cyclin D1 and survivin were significantly lower, and the levels of Bax was significantly higher, in the LV-GFP-E2F-1 group (all $P < 0.05$), and there was no statistically significant difference between LV-GFP-NC and PBS groups (Figure 7). The results suggest that overexpression of E2F-1 decreases



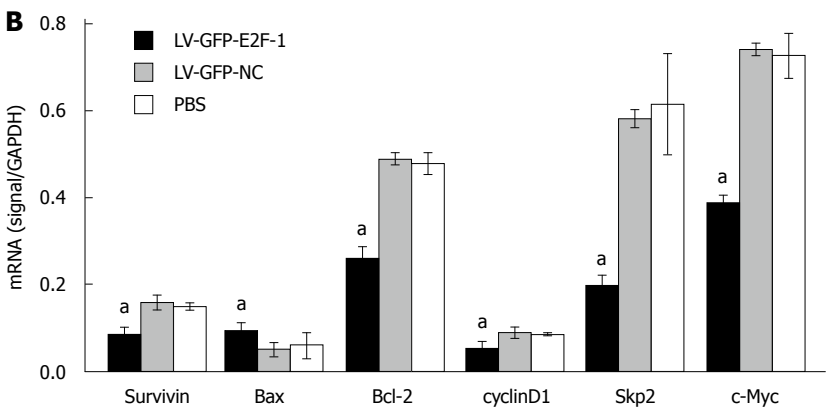


Figure 6 Overexpression of E2F-1 alters mRNA expression of apoptosis regulators. A: Agarose gel analysis of semiquantitative real-time polymerase chain reactions for c-Myc, S-phase kinase-associated protein 2 (Skp2), Bcl-2, cyclin D1, survivin, Bax and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in the MGC-803 tumor tissues. Lanes: 1, LV-GFP-E2F-1 group; 2, LV-GFP-NC group; 3, phosphate-buffered saline (PBS) group; M, 500 bp marker; B: Quantification showing downregulation of c-Myc, Skp2, Bcl-2, cyclin D1, survivin and upregulation of Bax mRNA levels (n = 8 animals for each condition). $^{a}P < 0.05$ using analysis of variance and Student-Newman-Keuls analyses.

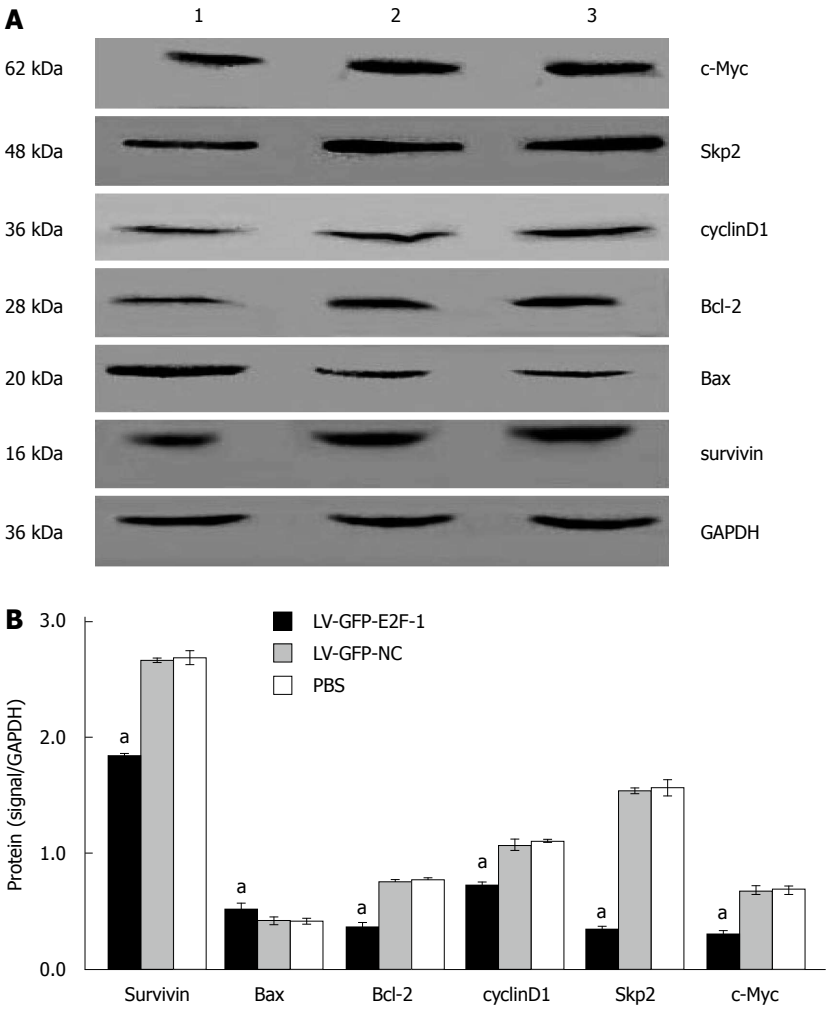


Figure 7 Overexpression of E2F-1 alters protein expression of apoptosis regulators. A: Western blot analysis of c-Myc, S-phase kinase-associated protein 2 (Skp2), Bcl-2, cyclin D1, survivin, Bax and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; internal control) in the MGC-803 tumor tissues. Lanes: 1, LV-GFP-E2F-1 group; 2, LV-GFP-NC group; 3, phosphate-buffered saline (PBS) group; B: Quantification showing downregulation c-Myc, Skp2, Bcl-2, cyclin D1, survivin and upregulation of Bax protein levels (n = 8 animals for each condition). $^{a}P < 0.05$ using analysis of variance and Student-Newman-Keuls analysis.

expression of c-Myc, Skp2, Bcl-2, cyclin D1 and survivin and increases Bax expression in MGC-803 tumors *in vivo*.

DISCUSSION

Cancer is a disease with dysregulation of the cell cycle and uncontrolled growth due to the combined effects of hereditary and environmental factors. The mechanism of cell cycle dysregulation is an important cause of the cell proliferation dropout that leads to cancer. Normally, each period of cell division and proliferation are strictly regulated by a variety of specific proteins.

The E2Fs are one of the most important cell cycle regulatory protein families[21]. Cell survival, mitosis, and apoptosis can be regulated by E2F family proteins[22]. E2F-1 is the most representative factor of this family, and it is generally considered to have the ability to induce apoptosis[23]. Many studies have shown that E2F-1 has anti-tumor effects in tumors such as colonic adenocarcinoma, glioma, and breast carcinoma[24-26]. However, E2F-1 also enhanced DNA replication and G1-to-S phase transition[27]. According to Conner *et al*[28], E2F-1 overexpression in the liver causes dysplasia and tumors. E2F-1 overexpression also enhances cell proliferation in epithelial ovarian cancer and may provide a useful prognostic indicator[29]. Expression of E2F-1 has been found in many human malignancies where it has two contrasting functions, including both inhibiting and promoting tumor growth. In addition, our previous studies have shown that downregulation of E2F-1 using RNAi successfully inhibits GC cell proliferation *in vivo* and *in vitro*[30,31]. It seems that E2F-1 is pleiotropic, and its functions are based on the dominant signaling pathway and cancer cell type.

In this study, MGC-803 cells were successfully inhibited by E2F-1 overexpression *in vivo*. This is consistent with our previous studies *in vitro*[15,16]. Therefore, lentiviral vector-mediated E2F-1 overexpression can be a new and effective treatment of GC. In this study, E2F-1 overexpression also decreased survivin, Bcl-2, cyclin D1, Skp2, and c-Myc expression, while Bax expression was increased. Thus, the *E2F-1* gene might affect GC growth

by modifying signaling pathways involving these genes.

The activation of mitochondrion-dependent processes is an effective approach for inducing apoptosis, which is regulated by the Bcl-2 family member. However, the proteins Bax and Bcl-2 have opposing roles in initiating mitochondrial apoptotic events and modulating apoptosis. Bcl-2 is associated with the outer mitochondrial membrane, where it plays a pivotal and important part in protecting mitochondrial structure and action, and prevents enzyme proteins from being released into the cytosol, thereby maintaining a stable intracellular environment[32]. In contrast, Bax, a dominant-negative inhibitor of Bcl-2, induces a mitochondrial permeability transition and promotes apoptosis. In this study, E2F-1 upregulated Bax and downregulated antiapoptotic Bcl-2, thereby elevating the ratio of Bax/Bcl-2 in MGC-803 cells. This suggests that cell apoptosis can be induced by a mitochondrion-dependent pathway, thereby activating caspase-9 and caspase-3[33]. Our current results are in good agreement with other recent studies, which demonstrated that E2F-1 induces apoptosis with Bax upregulation or Bcl-2 downregulation in melanoma cells[34] and human esophageal cancer cells[35].

Overexpression of E2F-1 downregulated the expression of c-Myc and Skp2, which are overexpressed in GC and promote tumor growth[36,37]. c-Myc is also involve in cell transcription and mitosis by management of cell cycle-related genes[38-41]. Zhang *et al*[42] confirmed that overexpression of c-Myc could enhance normal gastric cell growth and proliferation, as GC cell proliferation was inhibited by RNA interference targeting c-Myc gene silencing. Skp2 belongs to the family of F-box proteins. It has been known for a long time that Skp2 undergoes phosphorylation during cell cycle progression and growth factor stimulation[43,44]. Wei *et al*[45] reported that downregulation of Skp2 inhibited the growth and metastasis of GC cells *in vitro* and *in vivo*. Skp2 depletion also increased caspase-3 activity and impeded formation of filopodia and locomotion. Skp2 is associated with c-Myc transcription and degradation, and thus regulates Myc protein stability[46]. This establishes an unusual structured network that is the E2F-1/Skp2/c-Myc signaling pathway, by which E2F-1 suppresses MGC-803 cell growth *in vivo*.

Cyclin D1 is one of the most important proteins for cell cycle regulation, and is related to the development of many cancers and cancer cell growth. Cyclin D1 binds to and activates CDK4/6, which subsequently phosphorylates tumor-inhibiting protein Rb and promotes G1/S transition in the cell cycle[47,48]. So far, survivin is the strongest anti-apoptotic factor with the smallest molecular weight[49]. Survivin can also prevent tubulin from binding with spindle microtubules, and inhibit apoptosis in cell mitosis[50]. Furthermore, the proportion of cells in the G2/M phase were increased, while the number of cells in the G0/G1 phase decreased after transfecting survivin-specific siRNA into MGC-803 cells[51]. Thus, the proliferation of GC cells was inhibited, and apoptosis induced by survivin RNA interference. Dar *et al*[52] have shown that the proliferative capacity of melanoma cells is mediated by E2F-regulated Akt phosphorylation. Furthermore, the PTEN-Akt-p53-miR-365-Cyclin D1/cdc25A axis serves as a new mechanism underlying gastric tumorigenesis[53]. Akt signaling translationally regulates survivin expression for metastatic progression of colorectal cancer and GC[54,55].

Our present study has shown that E2F-1 overexpression significantly inhibited the growth of transplanted tumors and promoted cell apoptosis. E2F-1 overexpression also inhibited cyclin D1 and survivin expression, either directly or indirectly *via* the Akt signaling pathway, and suppressed MGC-803 cell growth *in vivo*. Therefore, E2F-1 plays an important role in GC cell survival, apoptosis, and mitosis. This research provides a basis for treatment of GC *via* regulation of E2F-1 expression.

COMMENTS

Background

Gastric cancer (GC) remains the second leading cause of death from malignant disease worldwide. Despite improvements in surgical techniques and the development of new chemotherapeutic regimens, the results are often disappointing in patients with advanced tumors. Gene therapy is a very hopeful approach to inhibit the proliferation of tumor cells and avoid the side effects of drug therapy. E2F-1, a member of the E2F family of transcription factors, is crucial for the E2F-dependent apoptotic program. However, few studies have reported on E2F-1 in GC. In particular, the effect and functional mechanism of E2F-1 overexpression in gastric cancer *in vivo* remain unclear.

Research frontiers

E2F-1 plays an important part in manipulating cell cycle progression and other cell biologic behaviors. E2F-1 can show antitumor or tumor-promoting effects, which remain controversial. The research hotspot of E2F-1 is how it affects the progression of human cancer.

Innovations and breakthroughs

Previous studies have indicated that the growth and proliferation of GC cells were inhibited and G1 to S phase cell cycle transition was blocked by E2F-1 overexpression *in vitro*. Recent reports were focused on its roles on *in vivo*. The authors discovered that E2F-1 inhibited tumor growth and promoted tumor cell apoptosis in models of human gastric carcinoma in nude mice. In addition, E2F-1 upregulation increased the expression of Bax and decreased expression of survivin, Bcl-2, cyclin D1, Skp2, and c-Myc expression level.

Applications

In understanding the role and mechanism of E2F-1-mediated multiple signaling pathways in GC *in vivo*, this study is expected to suggest a way to improve clinical treatment.

Terminology

E2F-1 is a transcription factor involved with DNA replication, cell survival, mitosis, and apoptosis. The retinoblastoma 1 (pRb) factor binds to E2F-1, and E2F-1 activation is determined by the state of pRb phosphorylation; E2F-1 is inhibited by dephosphorylated pRb and activated by pRb phosphorylation.

Peer review

This study is a very enjoyable and valuable work. The results may represent a molecular mechanism of E2F-1 in GC.

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ORIGINAL ARTICLE

Case Control Study

Hepatitis B virus infection, diabetes mellitus, and their synergism for cholangiocarcinoma development: A case-control study in Korea

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Abstract

AIM: To identify possible risk factors and their synergism for cholangiocarcinoma development.

METHODS: A hospital-based, case-control study in which we included 276 cholangiocarcinoma patients [193 extrahepatic cholangiocarcinoma (ECC) and 83 intrahepatic cholangiocarcinoma (ICC)], diagnosed at a training hospital in Korea between 2007 and 2013, and 552 healthy controls matched 2:1 for age, sex, and date of diagnosis. Risk factors for cholangiocarcinoma and possible synergism between those factors were evaluated using conditional logistic regression and synergism index, respectively.

RESULTS: There was an association between cholangiocarcinoma and hepatitis B virus (HBV) infection, diabetes mellitus (DM), cholecystolithiasis, choledocholithiasis, and hepatolithiasis, with the adjusted odds ratios (AORs) of 4.1, 2.6, 1.7, 12.4, and 39.9, respectively. Synergistic interaction on the additive model was investigated between HBV infection and DM (AOR = 12.2; 95%CI: 1.9-80.1). In the subgroup analyses, cholecystolithiasis, choledocholithiasis, hepatolithiasis, and DM were significant risk factors for ECC (AOR = 2.0, 18.1, 14.9, and 2.0, respectively), whereas choledocholithiasis, hepatolithiasis, HBV infection, and DM were risk factors for ICC (AOR = 8.6, 157.4, 5.3 and 4.9, respectively). Synergistic interaction was also observed between HBV infection and DM (OR = 22.7; 95%CI: 2.4-214.1). However, there was no synergistic interaction between other significant risk factors for cholangiocarcinoma.

CONCLUSION: In this Korean study, HBV infection and DM were found to exert independent and synergistic effects on the risk for cholangiocarcinoma, including ICC. Exploring the underlying mechanisms

for such synergy may lead to the development of cholangiocarcinoma prevention strategies in high-risk individuals.

Key words: Cholangiocarcinoma; Risk factor; Hepatitis; Synergism; Diabetes mellitus

Core tip: Although several risk factors for cholangiocarcinoma were identified in previous studies, details on their interactions or the influence of disease duration on the risk of cholangiocarcinoma are still unclear. Moreover, epidemiologic studies about cholangiocarcinoma in Korea are scarce. The present study in a Korean population showed that the impact of diabetes mellitus on the risk of cholangiocarcinoma was greater when diabetic complications were present. Further, it indicated that there was a synergistic effect between Hepatitis B virus infection and diabetes mellitus on the risk of cholangiocarcinoma, and that the synergistic effect was enhanced in cases of complicated diabetes.

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INTRODUCTION

Cholangiocarcinomas (CCAs) are highly fatal cancers of the biliary tract epithelium, which arise from intrahepatic [intrahepatic cholangiocarcinoma (ICC)] or extrahepatic bile ducts [extrahepatic cholangiocarcinoma (ECC)]. Although a rare malignancy, CCA is the second most common cancer of the liver[1]. Furthermore, the incidence of CCA has reportedly been increasing in several areas worldwide, especially the incidence of intrahepatic CCA[2-4]. Most CCAs are unresectable at presentation. Even after curative resection, 5-year survival rates of only 11%-44% have been reported[1]. Considering the poor prognosis and increasing incidence, it is crucial to recognize risk factors for CCA in order to decrease its incidence.

Several risk factors, including liver fluke infestation[5] and hepatolithiasis[6,7], were identified in East Asia including Korea, where CCA is more prevalent than in Western countries[8]. However, those account for < 30% of all CCA cases[1]. Recently, hepatitis B virus (HBV) infection[9] and diabetes mellitus (DM)[10] have been reported to be additional possible risk factors, but it has been estimated that only < 25% of CCA cases are related to these factors[7,11].

For the other primary liver cancer such as hepatocellular carcinoma, several synergistic effects between risk factors have been identified[12,13]. However, there have been little studies to focus on analyzing interactions between risk factors for CCA. Because of the multifactorial nature of biliary tract carcinogenesis, possible interactions between risk factors may exist. Therefore, we conducted a hospital-based case-control study to assess potential risk factors for CCA in Korea, and further evaluate possible synergisms between the risk factors identified.

MATERIALS AND METHODS

Study population

All patients diagnosed with CCA through pathological findings at the Cheju Halla General Hospital between January 2007 and April 2013 were reviewed for study enrollment. Pathological confirmation was based on definite cytology, small biopsy, or surgical pathology. Individuals diagnosed with other cancers before the date of CCA diagnosis were excluded from enrollment.

Control subjects, matched 2:1 with cases for age (± 3 years), sex, and date of diagnosis (± 3 mo), were randomly chosen among individuals who had visited the health screening center of the Cheju Halla General Hospital for a routine checkup during the same period as the CCA cases. We excluded subjects with diagnoses of cancers or who were missing any data regarding risk factors and cancers. Subjects without radiologic informations were also excluded. Finally, 276 cases and 552 controls were included for the analysis. The study protocol was approved by the Institutional Review Board of Cheju Halla General Hospital.

Data collection

Cases and controls were interviewed at the initial visit on their medical history, smoking, and alcohol use. Structured data collection sheets were routinely used in health screening center to obtain data on demographic and clinical characteristics. All eligible participants underwent radiological evaluations (abdominal ultrasound, computed tomography, and/or magnetic resonance cholangiopancreatography). Blood samples were also collected from all subjects at the time of initial examination.

All variables investigated for CCA risk evaluation were divided into 4 broad categories: biliary tract conditions, infectious etiologies, non-infectious liver diseases, and miscellaneous potential risk factors. Biliary tract conditions included cholecystolithiasis, choledocholithiasis, hepatolithiasis, cholecystectomy, primary sclerosing cholangitis, choledochal cyst, and liver fluke infestation. Non-infectious liver diseases included nonspecific liver cirrhosis and alcoholic hepatitis. The infectious diseases group included HBV infection and hepatitis C virus (HCV) infection. The miscellaneous potential risk factors included smoking, alcohol, obesity,

DM, thyroid disease, chronic pancreatitis, hypertension, and ulcerative colitis.

All data were obtained retrospectively from patient records. We only included information up to 1 year before the diagnosis of CCA for cases and 1 year before the cancer diagnosis of the index case for the matched controls.

Definitions of events

CCA was classified as either intrahepatic or extrahepatic CCA. Hilar CCA was included in ECC, and ampulla of Vater cancer was excluded in this analysis. A heavy drinker was defined as an individual currently drinking alcoholic beverages in a daily amount of $\geqslant$ 80 g (male) or $\geqslant$ 40 g (female)[14]. Obesity was defined as a body mass index of 25.0 kg/m^2 or greater, according to the Asian-Pacific criteria for obesity[15].

Blood samples were collected from cases and controls at the time of initial examination. Serum HBV surface antigen (HBsAg) and HCV antibody (anti-HCV) were assessed by using enzyme immunoassay (Abbott Laboratories, North Chicago, IL, United States), and anti-HCV-positive participants were tested for HCV RNA by using COBAS® Ampliprep (Roche Molecular Systems, Inc., CA, United States). HBV infection was defined as a positive hepatitis B surface antigen, and HCV infection was defined as a positive HCV RNA. The diagnostic criteria for cirrhosis were as follows: clinical manifestations of chronic hepatitis with portal hypertension (*e.g.*, collateral varices, varices, thrombocytopenia, or splenomegaly) and/or hepatic decompensation (*e.g.*, jaundice, prolonged prothrombin time, and ascites), laboratory tests, and radiologic studies. In patients undergoing surgical treatment, cirrhosis was also confirmed pathologically. Nonspecific cirrhosis was defined by the presence of cirrhosis without the presence of HCV, HBV, or alcoholic liver disease.

The diagnosis of liver fluke infestation was made on the basis of detection of ova or worms in feces, or radiologic finding of diffuse, uniform dilatation of the small intrahepatic bile ducts with no or minimal dilatation of larger bile ducts and with no focal obstructing lesion. Choledochal cysts were considered to be present if there was a characteristic cystic or fusiform dilatation of the extrahepatic or intrahepatic duct on radiologic findings. Choledocholithiasis was defined as the presence of at least one stone in the extrahepatic bile duct, whereas hepatolithiasis as the presence of stone in the intrahepatic bile duct. The presence of cystic duct stone was classified as cholecystolithiasis.

Diabetes was diagnosed according to the World Health Organization Criteria[16], and categorized into two groups: (1) complicated diabetes (presence of any stage of retinopathy, nephropathy or macrovascular complications); and (2) uncomplicated diabetes. Thyroid disease included hyperthyroidism and hypothyroidism.

Statistical analysis

Statistical analyses were performed by using SPSS 20.0 (SPSS incorporated, Chicago, IL, United States). The Mann-Whitney *U* test and the Pearson χ^2 with Fisher exact test were used to compare continuous and discrete variables, respectively. Univariate and multivariate analyses of correlation were carried out by using conditional logistic regression with maximum likelihood estimates of parameter values for assessing the risk for CCA. Among all variables investigated, primary sclerosing cholangitis, choledochal cyst, and nonspecific liver cirrhosis were not tested because cases were too few to be analyzed ($n < 3$ in whole study population including controls). All other variables were evaluated in the univariable conditional logistic regression analysis, and the variables with $P < 0.1$ in the univariate analysis were included in the multivariable models. The adjusted odds ratio (AOR) and 95%CI for each variable were estimated by using the logistic regression coefficient. In all analyses, $P < 0.05$ for two-sided tests was considered statistically significant.

The synergisms between risk factors were evaluated by including them in the additive regression model using an interaction term, since it is more appropriate to assess biological interactions and public health concerns. Multiple logistic regression models were used to evaluate departure from additivity. By crossing two independent risk factors for CCA, dummy variables of 4 categories were obtained; 2 for the presence of each risk factor alone, 1 for the presence of both risk factors, and 1 for the absence of both risk factors. The last of these categories was used as the reference category in the regression models. To assess the deviation from the additive model of no interaction between variables, the Synergism index (S) and its 95%CI, as proposed by Rothman, was calculated[17]; $S = (OR_{11} - 1)/(OR_{01} + OR_{10} - 2)$. OR_{10} and OR_{01} mean the OR for the presence of each risk factor in the absence of the other, whereas OR_{11} means the OR of the joint effect of two risk factors. A value of S equal to unity was interpreted as indicative of additivity, whereas a value greater than unity was indicative of superadditivity and synergism.

RESULTS

Patient characteristics

There were 276 patients with CCA eligible for this study. Out of these, 83 (30.1%) were ICC and 193 (69.9%) were ECC. The CCA patients and controls had a similar mean age (67.8 ± 12.5 *vs* 67.5 ± 12.5, $P = 0.818$) and proportion of men (50.4% *vs* 50.4%, male to female ratio, 1.02:1), suggesting that pairing was effective.

CCA population

The multivariate conditional logistic analysis showed that cholecystolithiasis (AOR = 1.74; 95%CI: 1.04-2.90), choledocholithiasis (AOR = 12.35; 95%CI: 4.31-35.38),

Table 1 Comparison of risk factors in patients with cholangiocarcinoma and matched controls *n* (%)

Variable	CCA patient (*n* = 276)	Control (*n* = 552)	Univariable analysis		Multivariable analysis	
			OR (95%CI)	*P* value	AOR (95%CI)	*P* value
Cigarette smoking	84 (30.4)	157 (28.4)	1.14 (0.79-1.66)	0.487	-	-
< 20 pack-years[1]	35 (12.7)	53 (9.6)	1.39 (0.85-2.27)	0.191	-	-
⩾ 20 pack-years	49 (17.8)	104 (18.8)	1.01 (0.66-1.55)	0.972	-	-
Heavy alcohol consumption[2]	35 (12.7)	50 (9.1)	1.53 (0.94-2.51)	0.088	1.45 (0.82-2.55)	0.199
Obesity[3]	64 (23.2)	134 (24.3)	0.94 (0.66-1.33)	0.722	-	-
Cholecystolithiasis	47 (17.0)	42 (7.6)	2.34 (1.52-3.61)	< 0.001	1.74 (1.04-2.90)	0.035
Choledocholithiasis	34 (12.3)	7 (1.3)	13.31 (5.20-34.07)	< 0.001	12.35 (4.31-35.38)	< 0.001
Hepatolithiasis	20 (7.2)	1 (0.2)	20.00 (4.68-85.57)	< 0.001	39.87 (7.25-219.17)	< 0.001
Cholecystectomy	17 (6.2)	23 (4.2)	1.49 (0.79-2.82)	0.216	-	-
Ulcerative colitis	2 (0.7)	3 (0.5)	1.33 (0.22-7.98)	0.753	-	-
Alcoholic liver disease	14 (5.1)	26 (4.7)	1.08 (0.55-2.13)	0.816	-	-
Thyroid disease	6 (2.2)	22 (4.0)	0.51 (0.20-1.31)	0.164	-	-
Chronic pancreatitis	1 (0.4)	5 (0.9)	0.40 (0.05-3.42)	0.403	-	-
Hypertension	113 (40.9)	254 (46.0)	0.80 (0.59-1.08)	0.150	-	-
Diabetes mellitus	65 (23.6)	69 (12.5)	2.22 (1.51-3.28)	< 0.001	2.55 (1.66-3.91)	< 0.001
Without complications	36 (13.0)	47 (8.5)	1.82 (1.12-2.96)	0.015	2.20 (1.30-3.70)	0.003
With complications[4]	29 (10.5)	22 (4.0)	2.98 (1.67-5.32)	< 0.001	3.25 (1.69-6.25)	< 0.001
HBV infection	28 (10.1)	18 (3.3)	3.34 (1.80-6.19)	< 0.001	4.12 (2.01-8.44)	< 0.001
HCV infection	11 (4.0)	13 (2.4)	1.69 (0.76-3.78)	0.199	-	-
Liver fluke infestation	6 (2.2)	4 (0.7)	3.00 (0.85-10.63)	0.089	3.49 (0.86-14.07)	0.079

[1]One pack-year = 1 pack per day for a year; [2]Daily amount of ⩾ 80 g (male) or ⩾ 40 g (female); [3]Obesity was defined as a body mass index > 25 kg/m^2 according to the Asian-Pacific criteria for obesity; [4]Any stage of retinopathy, nephropathy or macrovascular complications. CCA: Cholangiocarcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AOR: Adjusted odds ratio.

hepatolithiasis (AOR = 39.87; 95%CI: 7.25-219.17), HBV infection (AOR = 4.12; 95%CI: 2.01-8.44), and DM (AOR = 2.55; 95%CI: 1.66-3.91) were the significant risk factors for CCA (Table 1). HCV infection and heavy alcohol consumption were not significantly associated with development of CCA. When DM was dichotomized into complicated and uncomplicated DM, complicated DM resulted in a greater risk of CCA than uncomplicated DM (AOR = 3.25 and 2.20, respectively) (Table 1). However, there is no significant correlation between estimated AOR and duration of DM (AOR = 1.42 and 0.75 for 5-10 years and > 10 years, respectively, $P = 0.5$).

Subgroup analysis- ECC and ICC population

Subgroup analysis was performed to investigate risk factors for ECC and ICC development. We included 193 ECC patients and 386 controls, and 83 ICC patients and 166 controls in the conditional logistic regression model. When ECC and ICC cases were compared to their respective control participants, cholecystolithiasis, choledocholithiasis, hepatolithiasis, and DM were the significant risk factors for ECC (AOR = 2.01, 18.08, 14.87 and 1.99, respectively) (Table 2), whereas choledocholithiasis, hepatolithiasis, HBV infection, and DM were the significant risk factors for ICC development (AOR = 8.63, 157.37, 5.27, and 4.87, respectively) (Table 3). Cholecystolithiasis was the significant risk factor for ECC but not ICC development. However, DM was significantly associated with both ECC and ICC. As with the results in the entire CCA population, complicated DM also resulted in a greater risk of CCA than uncomplicated DM in both subgroup analyses, although AOR for uncomplicated DM did not reach statistical significance in the ECC population ($P = 0.055$) (Tables 2 and 3).

Interaction between risk factors

After evaluating the independent effects of each significant risk factor on CCA development, the inter-actions and synergism of those factors were investigated. Of all significant factors, hepatolithiasis was not included in this analysis because of the small number of cases and lack of controls, with hepatolithiasis and other significant risk factors together. Every pair of other significant risk factors was analyzed with adjustment for the rest of the significant factors. When investigating interactions between diabetes and HBV infection on the risk of CCA, the relative excess risk of developing CCA in patients having DM and HBV infection together exceeded the sum of the relative excess risks for each risk factor alone: 12.2-1.0 > (2.5-1.0) + (3.5-1.0). The estimated synergism index (S) was 2.80 (95%CI: 1.54-5.08), indicating the joint effect of DM and HBV infection is superadditive (Table 4, Figure 1A). When including only complicated diabetes instead of the entire diabetic cases in this analysis, the synergistic effect on the risk of CCA was greater than the effect between DM and HBV infection. The estimated synergism index (S) was 8.12 (95%CI: 4.92-13.38) (Table 4). These superadditivities of the joint effect between DM and HBV infection, or complicated DM and HBV infection were also investigated in the ICC subgroup population. The estimated synergism index (S) between DM and HBV infection, and complicated DM and HBV infection was 2.44 and 5.45, respectively (Table 5, Figure 1B). However, there was no synergistic interaction between other significant risk factors for CCA and ICC. Similarly, no significant interaction was observed between

Table 2 Comparison of risk factors in patients with extrahepatic cholangiocarcinoma and matched controls *n* (%)

Variable	ECC patient (*n* = 193)	Control (*n* = 386)	Univariable analysis		Multivariable analysis	
			OR (95%CI)	*P* value	AOR (95%CI)	*P* value
Cigarette smoking	53 (27.5)	91 (23.6)	1.32 (0.83-2.10)	0.240	-	-
< 20 pack-years	20 (10.4)	30 (7.8)	1.51 (0.79-2.87)	0.212	-	-
⩾ 20 pack-years	33 (17.1)	61 (15.8)	1.23 (0.73-2.08)	0.444	-	-
Heavy alcohol consumption	18 (9.3)	24 (6.2)	1.62 (0.83-3.17)	0.161	-	-
Obesity	42 (21.8)	90 (23.3)	0.91 (0.59-1.40)	0.658	-	-
Cholecystolithiasis	33 (17.1)	28 (7.3)	2.49 (1.48-4.20)	0.001	2.01 (1.12-3.58)	0.019
Choledocholithiasis	24 (12.4)	2 (0.5)	24.00 (5.67-101.55)	< 0.001	18.08 (4.18-78.19)	< 0.001
Hepatolithiasis	9 (4.7)	1 (0.3)	18.00 (2.28-142.08)	0.006	14.87 (1.79-123.74)	0.013
Cholecystectomy	13 (6.7)	19 (4.9)	1.38 (0.67-2.84)	0.376	-	-
Ulcerative colitis	2 (1.0)	1 (0.3)	4.00 (0.36-44.11)	0.258	-	-
Alcoholic liver disease	7 (3.6)	13 (3.4)	1.08 (0.42-2.78)	0.871	-	-
Thyroid disease	5 (2.6)	15 (3.9)	0.64 (0.22-1.84)	0.408	-	-
Chronic pancreatitis	1 (0.5)	5 (1.3)	0.40 (0.05-3.42)	0.403	-	-
Hypertension	84 (43.5)	185 (47.9)	0.83 (0.58-1.19)	0.301	-	-
Diabetes mellitus	44 (22.8)	54 (14.0)	1.88 (1.19-2.98)	0.007	1.99 (1.22-3.27)	0.006
Without complications	27 (14.0)	38 (9.8)	1.64 (0.95-2.85)	0.077	1.78 (0.99-3.19)	0.055
With complications	17 (8.8)	16 (4.1)	2.43 (1.18-5.00)	0.016	2.48 (1.16-5.32)	0.020
HBV infection	9 (4.7)	9 (2.3)	2.10 (0.80-5.49)	0.131	-	-
HCV infection	6 (3.1)	10 (2.6)	1.20 (0.44-3.30)	0.724	-	-
Liver fluke infestation	3 (1.6)	2 (0.5)	3.00 (0.50-17.95)	0.229	-	-

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ECC: Extrahepatic cholangiocarcinoma.

Table 3 Comparison of risk factors in patients with intrahepatic cholangiocarcinoma and matched controls *n* (%)

Variable	ICC patient (*n* = 83)	Control (*n* = 166)	Univariable analysis		Multivariable analysis	
			OR (95%CI)	*P* value	AOR (95%CI)	*P* value
Cigarette smoking	31 (37.3)	66 (39.8)	0.87 (0.47-1.63)	0.670	-	-
< 20 pack-years	15 (18.1)	23 (13.9)	1.17 (0.54-2.51)	0.689	-	-
⩾ 20 pack-years	16 (19.3)	43 (25.9)	0.68 (0.33-1.44)	0.318	-	-
Heavy alcohol consumption	17 (20.5)	26 (15.7)	1.44 (0.70-2.97)	0.319	-	-
Obesity	22 (26.5)	44 (26.5)	1.00 (0.56-1.80)	0.999	-	-
Cholecystolithiasis	14 (16.9)	14 (8.4)	2.06 (0.96-4.41)	0.062	1.04 (0.33-3.29)	0.941
Choledocholithiasis	10 (12.0)	4 (2.4)	6.20 (1.70-22.71)	0.006	8.63 (1.30-57.33)	0.026
Hepatolithiasis	11 (13.3)	1 (0.6)	22.00 (2.84-170.40)	0.003	157.37 (9.36-2646)	< 0.001
Cholecystectomy	4 (4.8)	4 (2.4)	2.00 (0.50-8.00)	0.327	-	-
Ulcerative colitis	0 (0.0)	2 (1.2)	0.03 (0.0-5748.1)	0.561	-	-
Alcoholic liver disease	7 (8.4)	13 (7.8)	1.09 (0.41-2.87)	0.868	-	-
Thyroid disease	1 (1.2)	7 (4.2)	0.25 (0.03-2.19)	0.211	-	-
Chronic pancreatitis	0 (0.0)	0 (0.0)	-	-	-	-
Hypertension	29 (34.9)	69 (41.6)	0.73 (0.41-1.31)	0.291	-	-
Diabetes mellitus	21 (25.3)	15 (9.0)	3.34 (1.60-7.01)	0.001	4.87 (1.88-12.59)	0.001
Without complications	9 (10.8)	9 (5.4)	2.52 (0.90-7.03)	0.078	4.00 (1.18-13.61)	0.027
With complications	12 (14.5)	6 (3.6)	4.28 (1.59-11.49)	0.004	6.13 (1.57-24.00)	0.009
HBV infection	19 (22.9)	9 (5.4)	4.58 (2.00-10.50)	< 0.001	5.27 (1.93-14.38)	0.001
HCV infection	5 (6.0)	3 (1.8)	3.33 (0.80-13.95)	0.099	1.71 (0.25-11.45)	0.582
Liver fluke infestation	3 (3.6)	2 (1.2)	3.00 (0.50-17.95)	0.229	-	-

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ICC: Intrahepatic cholangiocarcinoma.

significant risk factors for ECC. Only one ECC patient had DM and HBV infection, simultaneously.

DISCUSSION

The etiology and carcinogenesis of CCA remains obscure despite several established risk factors. In the present hospital based case-control study in Korea, we confirmed that HBV infection and DM were independent risk factors for CCA, particularly for ICC development, and found that there was a synergistic interaction between these factors regarding the risk of CCA. In the multivariate conditional logistic regression analysis, choledocholithiasis, hepatolithiasis, and DM were significantly associated with both ECC and ICC, whereas HBV infection and cholecystolithiasis were risk factors only for ICC and ECC development, respectively.

Our investigation of positive association between HBV infection and ICC was consistent with previous reports[7,18]. Previous studies demonstrated that both

Table 4 Interaction between diabetes mellitus and hepatitis B virus infection for cholangiocarcinoma: logistic regression analysis with adjusted odds ratio

Interaction variables		*n*	β Coefficient (± SE)	*P* value	AOR[1] (95%CI)	S (95%CI)[2]
DM	HBV					
Negative	Negative	658			1	
Positive	Negative	124	0.909 (0.22)	< 0.001	2.5 (1.6-3.8)	
Negative	Positive	36	1.259 (0.39)	0.001	3.5 (1.6-7.6)	
Positive	Positive	10	2.502 (0.96)	0.009	12.2 (1.9-80.1)	2.80 (1.54-5.08)
Complicated DM	HBV					
Negative	Negative	740			1	
Positive	Negative	42	0.967 (0.34)	0.005	2.6 (1.3-5.1)	
Negative	Positive	37	1.107 (0.38)	0.004	3.0 (1.4-6.4)	
Positive	Positive	9	3.423 (1.32)	0.009	30.7 (2.3-403.4)	8.12 (4.92-13.38)

[1]AOR: Odds ratio adjusted for the other significant risk factors for cholangiocarcinoma; [2]S = Synergy index described by Rothman = $(OR_{11} - 1)/(OR_{01} + OR_{10} - 2)$, where OR_{11} = OR of the joint effect of 2 risk factors; OR_{01} and OR_{10} = OR of each risk factor in the absence of the other. DM: Diabetes mellitus; HBV: Hepatitis B virus; AOR: Adjusted odds ratio.

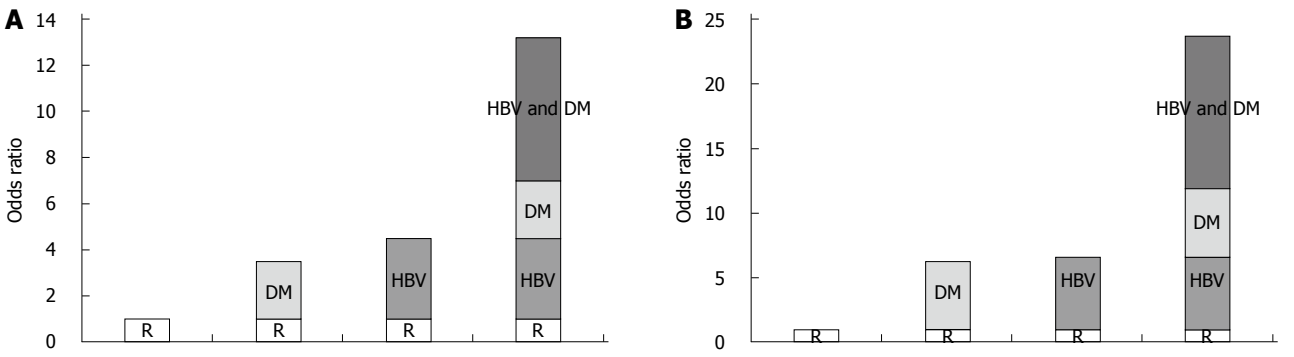

Figure 1 Risk of cholangiocarcinoma in subjects with diabetes mellitus, hepatitis B virus infection, or both. A: Whole cholangiocarcinoma population; B: Intrahepatic cholangiocarcinoma population. DM: Diabetes mellitus; HBV: Hepatitis B virus infection; R: Common reference category.

hepatocytes and cholangiocytes differentiate from the same hepatic progenitor cells; therefore, it is possible that HBV induces carcinogenesis in cholangiocytes through a similar mechanism as in hepatocytes[19,20]. In addition, HBV may be involved in the pathogenesis of ICC through a chronic inflammatory process[21,22]. Chronic inflammation of the biliary epithelium evoked by HBV infection can render it vulnerable to immunologic attack, leading to genetic alterations and subsequent malignant transformations of cells[23].

In contrast with HBV infection, HCV infection was not a significant risk factor for ICC in this investigation. This finding is in accordance with a previous Korean study[7]. Similarly, a recent meta-analysis did not identify a significant association between ICC development and HCV infection when analyzed in relation to East-Asian populations, whereas it did indicate a strong association (OR = 6.91) in relation to Western populations[18]. However, considering the small number of studies and participants analyzed, an additional large-scale study of Eastern regions is warranted to confirm the geographic variation.

Our results also indicated significant association between DM and CCA development, which is compatible with previous studies[7,18]. Insulin resistance and hyperinsulinemia have been shown to stimulate the growth of numerous cancer cell lines[24]. In addition, upregulated insulin-like growth factor 1 may stimulate liver cell proliferation, consequently leading to carcinogenesis of CCA[25,26].

For a more precise assessment, analyses were repeated after subclassifying all diabetic cases according to duration and the presence of complications. Although the duration of DM was not significantly correlated with the CCA risk, the impact of DM on the risk of CCA was greater when DM complications were present. Considering long time interval between actual DM onset and its clinical diagnosis[27], disease duration from diagnosis does not reflect the exact duration of illness. Moreover, control of DM and severity of the disease may be more crucial predictors than mere disease duration. Notably, occurrence of DM complications depends on glucose control and actual disease duration[28]. To clearly elucidate the impact of the duration of DM on the risk of CCA development, a future well-designed study with more detailed information is needed.

In this study, there were several interesting findings regarding the association between cholelithiasis and CCA: (1) cholecystolithiasis was a risk factor for ECC, but not ICC; (2) choledocholithiasis was a risk factor for both ECC and ICC; and (3) hepatolithiasis was a risk factor not only for ICC, but also for ECC. These results

Table 5 Interaction between diabetes mellitus and hepatitis B virus infection for intrahepatic cholangiocarcinoma: logistic regression analysis with adjusted odds ratio

Interaction variables		*n*	β Coefficient (± SE)	*P* value	AOR[1] (95%CI)	S (95%CI)
DM	HBV					
Negative	Negative	194			1	
Positive	Negative	27	1.670 (0.51)	0.001	5.3 (2.0-14.3)	
Negative	Positive	19	1.718 (0.58)	0.003	5.6 (1.8-17.4)	
Positive	Positive	9	3.120 (1.15)	0.006	22.7 (2.4-214.1)	2.44 (1.30-4.58)
Complicated DM	HBV					
Negative	Negative	211			1	
Positive	Negative	10	1.660 (0.77)	0.031	5.3 (1.2-23.8)	
Negative	Positive	20	1.528 (0.56)	0.006	4.6 (1.5-13.7)	
Positive	Positive	8	3.782 (1.51)	0.012	43.9 (2.3-849.5)	5.45 (3.16-9.42)

[1]AOR: Odds ratio adjusted for the other significant risk factors for intrahepatic cholangiocarcinoma. DM: Diabetes mellitus; HBV: Hepatitis B virus; AOR: Adjusted odds ratio; S: Synergism index.

may be explained by the effects of cholestasis, altered bile composition, and chronic proliferative inflammation near the stone-bearing ducts. Among the 9 patients with ECC who had hepatolithiasis in our study population, 7 patients had hilar cholangiocarcinoma, which supports this explanation. Previous Chinese studies showed the association between hepatolithiasis and ECC as well[29,30], which also supports our results.

The most noteworthy finding of this study is the synergistic effect between DM and HBV infection on the risk of CCA development. Although the definite mechanism is uncertain, there is a possible explanation for the interaction. Hyperglycemia could stimulate glucose oxidation, lipid peroxidation, and glycosylation of proteins, which leads to production of free radicals causing oxidative stress[31]. Oxidative stress subsequently may promote HBV gene expression, reactivation of viral replication, and liver disease chronicity, leading to DNA damage and CCA development[32,33]. Considering that oxidative stress is a widely accepted key mediator in the progression of DM and its complications[31,34], our finding of greater synergism between complicated DM and HBV infection support this explanation. It is considered that CCA and HCC share common etiologic factors, and a previous study on the risk factors for HCC also indicated the synergistic interaction between DM and HBV infection on cancer development[35,36], supporting our finding as well.

The present study has several potential limitations: (1) diagnostic bias cannot be excluded because cancer patients undergo additional testing, and thus may have more diagnoses than individuals without cancer; (2) this was a hospital-based study performed in a single institution, not a population-based design, therefore there is a possibility of selection bias caused by differential referral patterns; however, hospital-based design may be more appropriate for CCA in view of the low incidence and short survival of CCA patients; (3) hepatitis B core antibody (anti-HBc) and occult HBV infection were not investigated in this study; however, recent studies demonstrated very low levels of HBV DNA in subjects with anti-HBc alone (without surface antigen/antibody), and extremely low incidence of occult HBV infection[37,38]. Furthermore, the prevalence of HBV observed in the present study was comparable with the previous HBV prevalence estimates in Korea[39]; and (4) DM was not classified according to type; considering the absence of young-onset (≤ 30 years) DM in our cohort and extremely lower incidence of type I DM in Asia[40], most of the DM cases in this study were thought to be type 2 DM.

Despite these limitations, our study has several noteworthy strengths: (1) this is the first analysis of risk factors for ECC in Korea that was conducted after adjustment for possible confounding risk factors in a multivariable model. A previous Korean study[41] focused only on *Clonorchis sinensis* parasitosis as a risk factor for ECC without adjustment for confounders; (2) the prevalence of the significant risk factors in the control subjects was comparable with the prevalence in the general population of Korea[42,43] or other Asian country[44]; (3) we stratified biliary lithiasis and DM according to the location and the presence of the complication, respectively. To the best of our knowledge, this is the first study to investigate the impact of complicated and uncomplicated DM on CCA risk, after stratification of DM; and (4) most importantly, this is the first study to investigate the synergistic effect between DM and HBV infection on the risk of CCA development. This "new" finding may help stratify patients at risk for CCA and design CCA surveillance algorithms depending on the stratification.

In conclusion, besides the biliary lithiasis, HBV infection and DM were independent risk factors for CCA, especially for ICC development. In addition, there was synergistic interaction between the two factors on the risk for CCA development. A further large-scale study is warranted to confirm this synergistic interaction and to clarify possible underlying mechanisms. Exploring the underlying mechanisms for such synergy may lead to the development of CCA prevention strategies in high-risk individuals.

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COMMENTS

Background

Previous studies have identified several risk factors for cholangiocarcinoma (CCA) development. However, the etiology of CCA is still largely unknown. Moreover, the interaction between risk factors has not been investigated to date.

Research frontiers

Liver fluke infestation and hepatolithiasis were established as risk factors for CCA. In the recent investigations, hepatitis B virus (HBV) infection and diabetes mellitus (DM) were reported as significant risk factors for CCA as well.

Innovations and breakthroughs

In this study, the authors found that the impact of DM on the risk of CCA was greater when diabetic complications were present. The results showed that there was a synergistic effect between HBV infection and DM on the risk of CCA, and that the synergistic effect was enhanced in cases of complicated DM.

Applications

The authors' findings may help stratify patients at risk for CCA and design CCA surveillance algorithms depending on such stratification. Exploring the underlying mechanisms for synergy between HBV infection and DM may lead to the development of CCA prevention strategies in high-risk individuals.

Peer review

This is a well-designed and relevant study, showing that HBV infection and DM exert independent and synergistic effects on the risk for CCA, including intrahepatic CCA. The methodology is well described, and the statistics are sound. It makes a significant contribution to our understanding of the CCA etiology.

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ORIGINAL ARTICLE

Case Control Study

miRNA-103: Molecular link between insulin resistance and nonalcoholic fatty liver disease

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Abstract

AIM: To investigate the associations between miRNA-103 (miR-103) and insulin resistance and nonalcoholic fatty liver disease (NAFLD).

METHODS: Serum samples were collected from 50 NAFLD patients who were overweight or obese (NAFLD group) and from 30 healthy subjects who served as controls (normal control group). Quantitative polymerase chain reaction was used to detect expression of miR-103. Fasting plasma glucose, fasting insulin, and triglyceride (TG) levels were measured. Homeostasis model assessment was used to evaluate basal insulin resistance (HOMA-IR). Patient height and weight were measured to calculate body mass index (BMI).

RESULTS: Compared with the normal control group, higher serum levels of miR-103 were expressed in the NAFLD group (8.18 ± 0.73 *vs* 4.23 ± 0.81, $P = 0.000$). When $P = 0.01$ (bilateral), miR-103 was positively correlated with HOMA-IR ($r = 0.881$), TG ($r = 0.774$) and BMI ($r = 0.878$), respectively. miR-103, TG and BMI were all independent factors for HOMA-IR ($\beta = 0.438/0.657/0.251$, $P = 0.000/0.007/0.001$). miR-103, TG, BMI and HOMA-IR were all risk factors for NAFLD (odds ratio = 2.411/16.196/1.574/19.11, $P = 0.009/0.022/0.01/0.014$).

CONCLUSION: miR-103 is involved in insulin resistance and NAFLD, and may be a molecular link between insulin resistance and NAFLD and a therapeutic target for these disorders.

Key words: miRNA-103; Insulin resistance; Nonalcoholic fatty liver disease

Core tip: Insulin resistance activates development of nonalcoholic fatty liver disease (NAFLD), however, the molecular mechanism is not fully understood. We determined fasting plasma glucose, fasting insulin, triglyceride (TG) and the levels of miRNA-103 (miR-103) in the serum of patients with NAFLD. We found that higher levels of miR-103 were expressed in the serum of patients with NAFLD. miR-103 was positively correlated with homeostasis model assessment and was used to evaluate basal insulin resistance (HOMA-IR),

TG and BMI, respectively. miR-103 was an independent factor for HOMA-IR and a risk factor for NAFLD. We conclude that miR-103 is involved in insulin resistance and NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinical syndrome characterized by hepatic steatosis and fat deposition in hepatocytes in the absence of significant alcohol use. The incidence of NAFLD in the general Chinese population has almost doubled over the last 10-15 years and is highly prevalent in obese populations[1]. Obesity-associated insulin resistance is regarded as a factor that critically contributes to the development of NAFLD[2]. Insulin resistance resulting in a hyperinsulinemic state increases *de novo* lipogenesis, which further exacerbates hepatic lipid deposition and boosts the development of the disease.

miRNAs are endogenously expressed RNAs consisting of 20-24 nucleotides that affect the expression of hundreds of genes involved in numerous biological processes, including lipid metabolism, organ development, differentiation, brain morphogenesis, and apoptosis.

miRNAs are potent intracellular post-transcriptional regulators and are also selectively secreted into the circulation in a cell-specific fashion. miRNAs are now known to be stably expressed in serum[3], blood[4,5] and plasma[6]. Moreover, the unique expression patterns of these circulating miRNAs are related to specific human diseases[7]. miRNA-103 (miR-103) regulates insulin sensitivity and glucose homeostasis and is highly expressed in the liver of patients with NAFLD. Furthermore, there is a positive correlation between the patient's homeostatic model assessment (HOMA) index and miR-103 expression levels[8].

The clinical spectrum of NAFLD varies between steatosis with a benign clinical course and cirrhosis with serious complications, including hepatocellular carcinoma and liver failure, therefore, it is important to identify the molecular mechanisms and therapeutic targets of NAFLD. A "two-hit" mechanism has been proposed, however, the underlying molecular mechanism is not fully understood. In this study, we aimed to determine the levels of miR-103 expressed in the serum of patients with NAFLD to explore the associations between miR-103 and insulin resistance and NAFLD in order to identify new molecular therapeutic targets for these disorders.

MATERIALS AND METHODS

Subjects

This study enrolled a cohort of 50 patients with NAFLD who were treated at the Department of Endocrinology of the Second Affiliated Hospital of Medical College of Qingdao University, China from November 2011 to April 2013. Thirty age-matched healthy subjects were selected as controls. Blood pressure and electrocardiogram findings were normal in all patients. Patients with NAFLD were newly diagnosed and had not received any treatment. The diagnosis of NAFLD was based on the presence of an ultrasonographic pattern consistent with "bright liver" (brightness and posterior attenuation) with stronger echoes in the hepatic parenchyma than in the renal parenchyma, vessel blurring, and narrowing of the lumen of the hepatic veins in the absence of findings suggestive of other chronic liver disease. All cases of fatty liver were in accordance with the Chinese diagnostic criteria for NAFLD (alcohol consumption < 40 g per week and without consideration of alteration in liver enzymes). Body mass index (BMI) was calculated based on the following formula: BMI = weight/height^2. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Medical College of Qingdao University.

Measurements

Fasting plasma glucose (FPG), fasting insulin (Fins), triglyceride (TG), and miR-103 levels were measured. Blood samples were obtained after an 8-h fasting period. Fins was measured by radioimmunoassay (RuiQi Biotechnology Corporation, Shanghai, China) with a sensitivity of 2 mU/L (normal range 0.5-25 mU/L). The insulin resistance index [homeostasis model assessment-insulin resistance (HOMA-IR)] was calculated using the HOMA model: (fasting insulin × fasting glucose)/22.5[9]. The expression of miR-103 was detected using real-time PCR.

Microarray profiling of serum miRNA

Total RNA was extracted from normal controls and patients with NAFLD (Biological Technology Co. Ltd., Chengdu, China). miRNA microarray analyses were carried out by Biological Technology using an ABI3730 Sequencer (Applied Biosystems, United States).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, United States). Normally distributed data were expressed as mean ± SD. The *t* test was used to compare groups in the study. Pearson correlation analysis and stepwise regression analysis were used for simple correlation and multivariate analysis, respectively. $P < 0.05$ was considered statistically significant.

Table 1 Clinical characteristics of the study subjects (mean ± SD)

	NAFLD group (n = 50)	Normal control group (n = 30)
Age (yr)	50 ± 6.70	50 ± 6.81
Gender (male/female)	28/22	16/14
BMI (kg/m^2)	28.70 ± 3.12[b]	22.80 ± 3.07
ln (HOMA-IR)	1.72 ± 0.35[b]	0.38 ± 0.31
Fins (IU/mL)	16.3 ± 3.06[b]	8.55 ± 3.21
TG (mmol/L)	2.67 ± 1.23[b]	1.58 ± 1.19
TC (mmol/L)	5.01 ± 0.89[a]	4.61 ± 0.97
FPG (mmol/L)	5.95 ± 0.93[b]	4.85 ± 0.87
ALT (U/L)	38.10 ± 19.80[b]	26.30 ± 20.14

[a]P < 0.05, [b]P < 0.01 *vs* the control group (unpaired Student t test and χ^2 test). NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; Fins: Fasting insulin; ln (HOMA-IR): Homeostasis model assessment-insulin resistance was log-transformed before analysis as it was not normally distributed; FPG: Fasting plasma glucose; TC: Total cholesterol; TG: Triglycerides; ALT: Alanine transaminase.

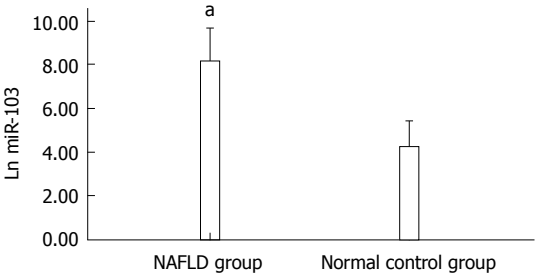

Figure 1 Differential expression of microRNA-103 levels in the serum of patients with nonalcoholic fatty liver disease. microRNA (miR)-103 was log-transformed before analysis because it was not normally distributed. [a]P < 0.05 *vs* normal control group (unpaired Student t test). NAFLD: Nonalcoholic fatty liver disease.

RESULTS

General characteristics

A total of 80 cases were included in this study. With the exception of age and gender (P > 0.05), BMI, HOMA-IR, Fins, TG, total cholesterol (TC), FPG, and alanine aminotransferase (ALT) in patients with NAFLD were higher than those in healthy controls (P < 0.05) (Table 1).

Differential expression of miR-103 levels in serum of patients with NAFLD

The levels of miR-103 expressed in the serum of NAFLD patients were higher than those in healthy controls (8.18 ± 0.73 *vs* 4.23 ± 0.81, P < 0.05) (Figure 1).

Pearson correlation analysis of associations between miR-103 and HOMA-IR, BMI and TG

Positive correlations were observed between miR-103 and HOMA-IR (r = 0.881), BMI (r = 0.878) and TG (r = 0.774) (Figure 2).

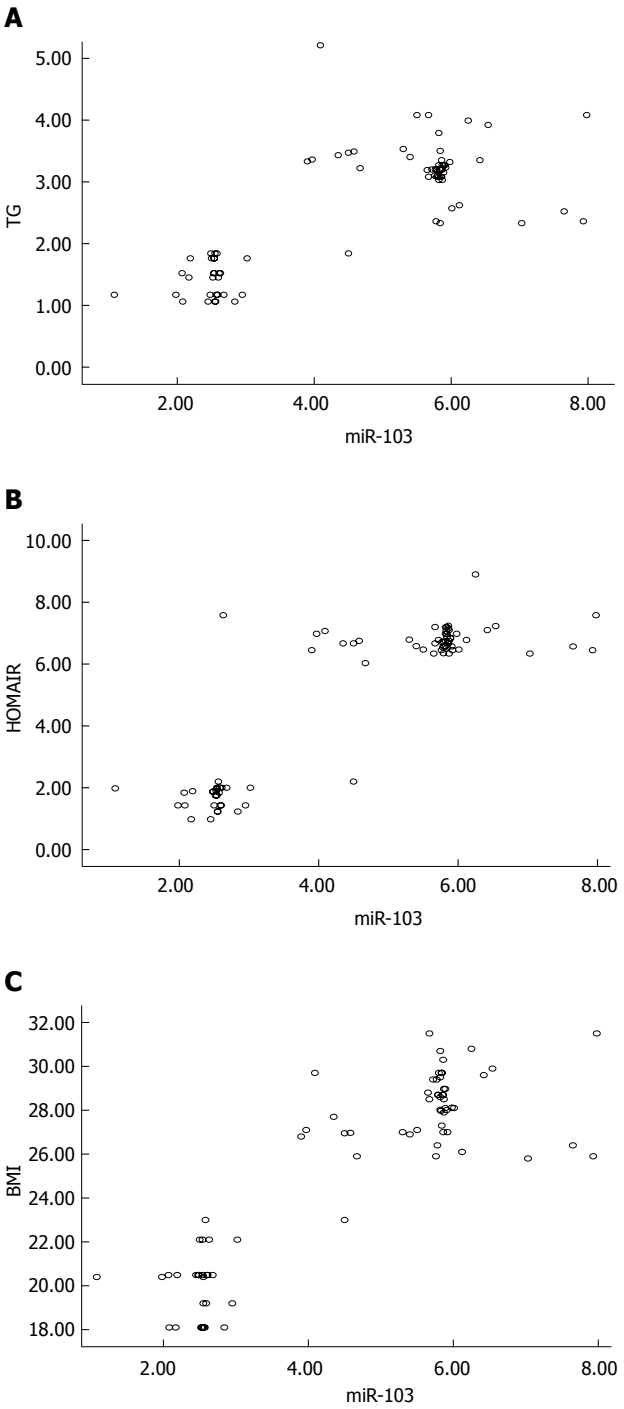

Figure 2 Correlation between microRNA-103 and Triglycerides (A), Homeostasis model assessment-insulin resistance (B), and body mass index (C). A: microRNA (miR)-103 was positively correlated with Triglycerides (TG) (r = 0.774, P = 0.01, bilateral); B: miR-103 was positively correlated with Homeostasis model assessment-insulin resistance (HOMA-IR) (r = 0.881, P = 0.01, bilateral); C: miR-103 was positively correlated with body mass index (BMI) (r = 0.878, P = 0.01, bilateral).

Multivariate analysis of miR-103, BMI, TG and HOMA-IR

HOMA-IR was a dependent variable and miR-103, TG and BMI were independent variables. Multivariate

Table 2 Linear regression analysis of factors affecting homeostasis model assessment-insulin resistance

	Independent variables	Independent variables	SE standard regression	*t* value	*P* value
BMI	0.251	0.072	0.431	3.510	0.001
miR-103	0.438	0.113	0.302	3.890	0.000
TG	0.657	0.236	0.258	2.779	0.007

BMI: Body mass index; miR-103: MicroRNA-103; TG: Triglyceride; HOMA-IR: Homeostasis model assessment-insulin resistance.

Table 3 Binary logistic regression analysis of factors affecting nonalcoholic fatty liver disease

Variables	*P* value	OR	95%CI
miR-103	0.009	2.411	1.25-4.652
TG	0.022	16.196	1.507-174.013
BMI	0.010	1.574	1.117-20.219
HOMA-IR	0.014	19.11	1.808-202.001

BMI: Body mass index; miR-103: MicroRNA-103; TG: Triglyceride; HOMA-IR: Homeostasis model assessment-insulin resistance.

linear regression analyses showed that miR-103, TG and BMI were all independent factors for HOMA-IR (β = 0.438/0.657/0.251, P = 0.000/0.007/0.001) (Table 2).

Binary logistic regression analysis of miR-103, BMI, TG, HOMA-IR and NAFLD

NAFLD was a dependent variable and miR-103, TG, BMI and HOMA-IR were independent variables. Binary logistic regression analysis showed that miR-103, TG, BMI and HOMA-IR were all risk factors for NAFLD (OR = 2.411/16.196/1.574/19.11, P = 0.009/0.022/0.01/0.014) (Table 3).

DISCUSSION

In this study, we found that HOMA-IR, Fins, TG, and FPG levels in patients with NAFLD were higher than those in healthy controls. These results suggest that these higher levels exist in patients with insulin resistance, and lipid and glucose abnormalities. To date, despite significant efforts, the accurate pathogenesis of NAFLD is not fully understood.

NAFLD, the prevalence of which is increasing in obesity, is one of the most frequent causes of chronic liver diseases and is characterized by the accumulation of lipids in hepatic cells. It is closely associated with hypertriglyceridemia, insulin resistance and intestinal microbiota changes[10,11]. More specifically, the input of lipid exceeds the output of lipid from the liver, which induces storage of lipid in the liver contributing to the development of hepatic steatosis. According to the two-hit hypothesis, insulin resistance results in increased intrahepatic triglyceride accumulation and this is the first hit, followed by the second step. The latter likely involves cytochrome P450 activation, oxidative stress, increased inflammatory cytokine production, lipid peroxidation, activation of hepatic stellate cells, and apoptosis. França *et al*[12] found that hypertriglyceridemia and liver steatosis were associated with increased microsomal triglyceride transfer protein expression. Another study reported that phospholipid ω-3 polyunsaturated fatty acids may play an important role in the development of NAFLD[13]. Therefore, many of the mechanisms underlying this association are still unclear.

Insulin resistance, described as the inability of insulin to stimulate glucose uptake, is a risk factor for the development of NAFLD[14]. Insulin resistance results in a reduction in lipolysis inhibition by insulin, which leads to fatty acid accumulation contributing to altered mitochondrial function, increased lipid intermediates and hepatic steatosis[15]. Insulin activates sterol regulatory element-binding protein (SREBP)1c, a master regulatory transcription factor in lipid synthesis, through stimulation of the mammalian target of rapamycin complex 1, which leads to increased lipogenesis[16]. Therefore, insulin resistance characterized by a hyperinsulinemic state as observed in patients with NAFLD increases *de novo* lipogenesis, which further exacerbates hepatic lipid deposition and accelerates development of the disease.

Recent studies have indicated that miRNAs are involved in the development of NAFLD, and serum levels of miRNAs are correlated with the severity of liver steatosis[17,18] and may represent novel, noninvasive biomarkers of diagnosis and histological disease severity in patients with NAFLD[19,20]. Hoekstra *et al*[21] reported that fatty liver development in low-density lipoprotein receptor knockout mice was associated with a significant change in the hepatocyte miRNA profile, a fivefold decrease in miR-302a expression was reported, which predisposed the liver to insulin resistance.

miR-103 results in insulin resistance. Trajkovski *et al*[8] reported that silencing of miR-103 led to improved insulin resistance. In contrast, gain of miR-103 function in either liver or fat was sufficient to induce insulin resistance. Further studies confirmed that high expression of miR-103 led to insulin resistance by downregulating caveolin-1, which is the direct target gene of miR-103 and a critical regulator of the insulin receptor. In addition, silencing of miR-103 decreased total fat by reducing adipocyte size. Furthermore, adiponectin levels were increased in anti-miR-103-injected *ob/ob* mice. Smaller adipocytes were associated with increased insulin sensitivity in human and rodent models[22], and adiponectin levels were positively correlated with insulin sensitivity[23]. In our study, we also found that miR-103 was positively correlated with HOMA-IR and was an independent

factor in overweight or obese patients with NAFLD. These data indicate that miR-103 is indirectly involved in the development of NAFLD due to insulin resistance.

Increased hepatic expression of miR-103 is directly involved in the development of NAFLD. NAFLD is highly associated with obesity and insulin resistance and is accompanied by hypertriglyceridemia, histologically characterized by hepatic TG accumulation of > 5%, resulting in steatosis. Xie *et al*[24] reported that miR-103 accelerates adipogenesis when expressed ectopically. miR-103 was increased in the liver of patients with NAFLD[8]. Our results showed that NAFLD patients had higher levels of miR-103 expression in serum compared with normal controls, and miR-103 was positively correlated with TG. Taken together, these results indicate that high expression of miR-103 may be directly involved in the development of NAFLD by increasing adipogenesis in hepatocytes leading to ectopic lipid deposition, thus contributing to hepatic steatosis.

miR-103 links insulin resistance and NAFLD. It has been reported that BMI and TG are the main factors related to the severity of NAFLD[25] and TG is regarded as an independent parameter associated with NAFLD[26]. Our results showed that miR-103 was positively correlated with HOMA-IR, TG and BMI, respectively, and miR-103, TG and BMI were all independent factors associated with HOMA-IR. MiR-103, TG, BMI and HOMA-IR were all risk factors for NAFLD. Therefore, miR-103 may be a potential molecular link between insulin resistance and NAFLD.

In conclusion, our results indicated that high expression of miR-103 directly increases adipogenesis in hepa-tocytes and indirectly results in hypertriglyceridemia due to insulin resistance, thus contributing to the development of NAFLD. Therefore, miR-103 is involved in insulin resistance and NAFLD, and may be regarded as a potential molecular link between them and a therapeutic target of these disorders. However, miR-103 as the bridge between insulin resistance and NAFLD requires further evidence. As our study included a small sample size and no liver biopsies were obtained, the above results require to be confirmed in a larger study which includes liver biopsies.

COMMENTS

Background

The pathogenesis of nonalcoholic fatty liver disease (NAFLD) remains obscure. Insulin resistance activates the development of NAFLD, however, the molecular mechanisms involved are not fully understood. miRNAs are involved in the development of NAFLD, and are known to be stably expressed in serum, blood and plasma. Moreover, the unique expression patterns of these circulating miRNAs are correlated with specific human diseases. miRNA-103 (miR-103) regulates insulin and glucose homeostasis and is highly expressed in the liver of patients with NAFLD.

Research frontiers

Insulin resistance activates the development of NAFLD, and miR-103 regulates insulin sensitivity. miRNAs are involved in the development of NAFLD, and serum levels of miRNAs are correlated with the severity of liver steatosis and may represent novel, noninvasive biomarkers of diagnosis and histological disease severity in patients with NAFLD.

Innovations and breakthroughs

Previous studies have shown that miR-103 regulates insulin sensitivity in obese mice and is increased in the liver of patients with NAFLD. In this study, the authors determined fasting plasma glucose, fasting insulin, triglyceride (TG) and expressed miR-103 levels in the serum of patients with NAFLD. The levels of miR-103 expressed in serum were higher in patients with NAFLD than in controls. miR-103 was positively correlated with homeostasis model assessment-insulin resistance (HOMA-IR), TG and body mass index (BMI), respectively. miR-103 was an independent factor of HOMA-IR and a risk factor for NAFLD. Therefore, miR-103 is involved in insulin resistance and NAFLD and may be regarded as the link between them and a therapeutic target in both disorders.

Applications

The study results suggest that high expression of miR-103 directly increases adipogenesis in hepatocytes and indirectly results in hypertriglyceridemia due to insulin resistance, thus contributing to the development of NAFLD. miR-103 is involved in insulin resistance and NAFLD, and may be regarded as a potential molecular link between them and a therapeutic target in both disorders.

Terminology

Insulin resistance is a physiological condition in which cells fail to respond to the normal actions of the hormone insulin. The body produces insulin, but the cells in the body become resistant to insulin and are unable to use it effectively, leading to hyperglycemia. Pancreatic β cells subsequently increase their production of insulin, further contributing to hyperinsulinemia, which is involved in the development of type 2 diabetes, hypertension, NAFLD and cancer. miR-103 belongs to the family of miRNAs, which are noncoding, highly conserved regulatory RNAs, which help to regulate gene expression at the post-transcription level. miR-103 is involved in the development of insulin resistance, cancer, and other diseases.

Peer review

This was an interesting study showing the relationship between miR-103, insulin resistance and NAFLD and an interesting paper reporting novel findings regarding the role of miR-103 in the pathogenesis of NAFLD, but miR-103 as the bridge of insulin resistance and NAFLD, may need more evidence.

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ORIGINAL ARTICLE

Retrospective Study

Assessment of liver ablation using cone beam computed tomography

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Abstract

AIM: To investigate the feasibility and accuracy of cone beam computed tomography (CBCT) in assessing the ablation zone after liver tumor ablation.

METHODS: Twenty-three patients (17 men and 6 women, range: 45-85 years old, mean age 65 years) with malignant liver tumors underwent ultrasound-guided percutaneous tumor ablation [radiofrequency (n = 14), microwave (n = 9)] followed by intravenous contrast-enhanced CBCT. Baseline multidetector computed tomography (MDCT) and peri-procedural CBCT images were compared. CBCT image quality was assessed as poor, good, or excellent. Image fusion was performed to assess tumor coverage, and quality of fusion was rated as bad, good, or excellent. Ablation zone volumes on peri-procedural CBCT and post-procedural MDCT were compared using the non-parametric paired Wilcoxon t-test.

RESULTS: Rate of primary ablation effectiveness was 100%. There were no complications related to ablation. Local tumor recurrence and new liver tumors were found 3 mo after initial treatment in one patient (4%). The ablation zone was identified in 21/23 (91.3%) patients on CBCT. The fusion of baseline MDCT and peri-procedural CBCT images was feasible in all patients and showed satisfactory tumor coverage (at least 5-mm margin). CBCT image quality was poor, good, and excellent in 2 (9%), 8 (35%), and 13 (56%), patients respectively. Registration quality between peri-procedural CBCT and post-procedural MDCT images was good to excellent in 17/23 (74%) patients. The median ablation volume on peri-procedural CBCT and post-procedural MDCT was 30 cm^3 (range: 4-95 cm^3) and 30 cm^3 (range: 4-124 cm^3), respectively (P-value > 0.2). There was a good correlation (r = 0.79) between the volumes of the two techniques.

CONCLUSION: Contrast-enhanced CBCT after tumor ablation of the liver allows early assessment of the ablation zone.

Key words: Ablation; Cone beam computed tomography; Liver; Malignancies; Radiofrequency

Core tip: Immediate intravenous contrast-enhanced cone beam computed tomography after percutaneous tumor ablation of the liver provides early assessment of the ablation zone and may provide the same information as multidetector computed tomography (MDCT) performed 1-2 mo after ablation. This is particularly interesting for centers that do not have MDCT in interventional rooms.

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INTRODUCTION

Local ablative techniques have become increasingly popular for the treatment of small malignant tumors of the liver, especially hepatocellular carcinoma (HCC) and liver metastases. Radiofrequency ablation (RFA) is the most widely used of these methods[1]. Microwave ablation (MWA) is another technique that has become increasingly popular since it does not require the conduction of electricity into tissue and it is not limited by charring[2]. Its efficacy has been shown to be similar to RFA in local tumor control[3,4].

One of the main drawbacks of local ablation is local intrahepatic recurrence. The most common risk factors for local tumor progression are tumor size, proximity to a major vessel, subcapsular location, and an insufficient ablative safety margin[5-7]. The latter factor seems to play a major role because significant agreement (86.8%) was found between the exact sites of local tumor progression and an insufficient ablative margin[8].

Procedure guidance methods include ultrasound, computed tomography (CT) scan and, more rarely, magnetic resonance imaging[9-11]. Depending on the institution, the patient characteristics, and the experience of the interventional radiologists, percutaneous tumor ablation can be guided by ultrasound only, CT scan only, or both. In the latter case, ultrasound is usually used for initial probe placement and CT to assess tumor coverage.

Cone beam computed tomography (CBCT) has been mainly evaluated for vascular applications such as transarterial chemoembolization[12-14], but also for non-vascular applications, such as image-guided procedures of the spine and pelvis[15]. Morimoto *et al*[16] described their initial experience in the liver in five patients who underwent radiofrequency ablation for malignant tumors, in which CBCT was used for needle placement but not to assess tumor coverage. More recently, Iwazawa *et al*[17,18] compared the effectiveness of CBCT and contrast-enhanced CT in assessing ablation margins. The authors concluded that the techniques were nearly equivalent. Because of the advantages of US-guided procedures and limitations of these techniques in monitoring tumor coverage, we hypothesized that CBCT could be used to assess effective ablation, especially in centers without CT dedicated to interventional radiology.

Thus, the purpose of this study was to evaluate the feasibility and accuracy of CBCT in assessing the ablation zone immediately after US-guided percutaneous tumor ablation with either RFA or MWA compared to pre-procedural CT and post-procedural CT 1-2 mo after ablation.

MATERIALS AND METHODS

Patients and tumor characteristics

Institutional review board approval was obtained for this retrospective study and informed consent was waived. Inclusion criteria were: (1) the presence of a solitary malignant liver tumor treated percutaneously < 40 mm in diameter; (2) RFA or MWA performed in the interventional radiology room with CBCT acquisition immediately after tumor ablation; and (3) available pre- and 1-2 mo post-procedural contrast-enhanced CT scan examinations.

Between June 2009 and September 2012, 29 patients were identified. Six of these patients were excluded: three were lost to follow-up and another three could not receive iodine intravenous contrast agents. Thus, the final patient population included 23 patients (17 men and 6 women, range 45-85 years old, mean age 65 years old). There were 17 patients with HCC. Twelve patients presented with treatment-naïve HCC while five others had new tumors following either liver resection (n = 2), or transarterial chemoembolization (n = 3). Four patients had recurrent liver metastases following liver resection (3 colorectal and 1 breast). Two patients had recurrent intrahepatic cholangiocarcinoma following liver resection. The median tumor size was 20 mm (range: 8-40 mm). The median follow-up was 10.8 mo (range: 2-33 mo).

The routine protocol included a baseline multidetector CT (MDCT) scan examination performed before percutaneous tumor ablation for future comparison. A CT scan examination was performed between 1-2 mo after tumor ablation, every 3 mo for 1 year, and every 6 mo thereafter.

Helical multiphasic CT examinations were performed using a 64-section MDCT scan (CT Light-Speed Ultra; GE Healthcare, Milwaukee, WI, United States). All examinations included an unenhanced liver acquisition, and arterial, portal venous, and delayed phase images at 30-35, 70, and 180 s, respectively, after an intravenous injection of a 2 mL/kg dose of non-ionic contrast material (Iobitridol; Xenetix®, Guerbet, Aulnay-sous-bois, France), administered at a rate of 4 mL/s with a mechanical power injector (Medrad; Pittsburgh, PA, United States). Reconstruction slice thickness was 1.25 mm.

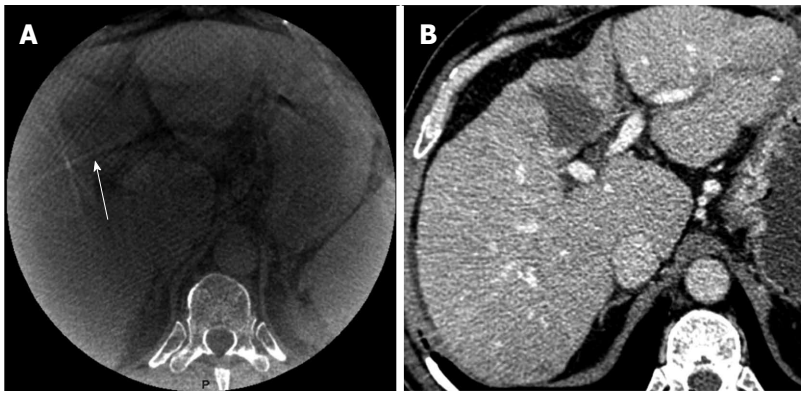

Figure 1 Illustration of a poor quality cone beam computed tomography image obtained in a 65-year-old man with hepatocellular carcinoma on hepatitis C virus cirrhosis background. A: Visibility of the ablation zone is poor (arrow); B: Post-ablation multi-detector computed tomography performed at 6 wk showed complete ablation.

Tumor ablation

For each procedure, coagulation parameters were within the normal values. All RF (n = 14) and microwave (n = 9) ablations were performed percutaneously by one of two radiologists (8 and 10 years of experience) in the angiography room using real-time ultrasound guidance on an inpatient basis. Patients received conscious sedation with fentanyl citrate and propofol. Local anesthesia was achieved by injecting 5 mL of 1% lidocaine hydrochloride.

RF procedures were performed with a 15-gauge expandable electrode (Talon-Star-Burst; RITA Medical System, Mountain View, CA, United States) for monopolar (n = 5), and with internally cooled electrodes (Prosurge; Celon/Olympus, Berlin, Germany) for multiprobe (n = 9) procedures. Microwave procedures were performed with a 14-gauge cooled-shaft antenna (Aculis; Microsulis Medical Limited, Denmead, Hampshire, United Kingdom) in the remaining patients. Single-session ablations were performed depending on the tumor size and manufacturer's recommendations, and included a 5-mm margin of normal-appearing hepatic tissue surrounding the tumor.

Cone beam CT acquisition technique

CBCT was performed at the end of the tumor ablation procedure. Electrodes were retracted to avoid metallic artifacts. If tumor coverage was satisfactory on 3D images, electrodes were removed and the needle track was treated by thermocoagulation.

CBCT was performed during breath holding, after elevating the patients' arms above the head on a large format digital flat-panel angiographic system (GE-Healthcare Innova™ 4100IQ; Chalfont St Giles, United Kingdom). In the first 14 patients, the CBCT acquisitions took 10 seconds (10 s group) while in the remaining 9 patients CBCT rotation speed was decreased and acquisitions were obtained during a 20-s rotation protocol (20 s group). Acquisition started 70 s after intravenous administration of 90 mL of a non-ionic contrast agent (Iobitridol; Xenetix®, Guerbet, Aulnay-sous-bois, France), administered at a rate of 3 mL/s with a mechanical power injector (Medrad; Pittsburgh, PA, United States). No arterial phase acquisitions were obtained.

Reconstructed images were automatically forwarded to a specific workstation (Innova3DXR software and Advantage Workstation; GE Healthcare, Chalfont St Giles, United Kingdom) through the local network for analysis using various renderings of 3D images, such as volume rendering, maximum intensity projection, and multi-planar reformations.

Image analysis

The following analysis of images was retrospectively performed by two experienced radiologists blinded to the results, and a consensus was obtained: (1) evaluation of CBCT image quality; (2) assessment of technical success, *i.e.*, tumor coverage at the end of tumor ablation; and (3) comparison of ablation zones with CBCT with 1-2 mo post-procedural MDCT data.

Image quality of cone beam CT: The quality of CBCT images was evaluated on a semi-quantitative scale ranging from 1-3 based on detection of the ablation zone and the presence of artifacts as follows: (1) poor: no visible ablation; (2) good: detection of ablation but the presence of artifacts preventing a clear definition of the zone; and (3) excellent: detection of the ablation zone and no artifacts. Figure 1 illustrates a poor image quality.

Assessment of tumor coverage: Baseline MDCT and peri-procedural CBCT images were compared and tumor coverage was assessed using multimodality image fusion performed with commercially available software (Integrated Registration; GE Healthcare, Chalfont St Giles, United Kingdom). The quality of image fusion was rated in relation to the contours of the liver and vessels by an experienced radiologist as follows: (1) bad registration (> 20 mm for liver contours); (2) good registration (between 5-20 mm for both liver vessels and contours); and (3) excellent registration for both liver vessels and contours (< 5 mm for both liver vessels and

contours). Tumor coverage was defined as partial if portions of the tumors could be seen outside the hypo-attenuating ablation zone, complete if the entire tumor was located within the hypo-attenuating ablation zone, and satisfactory if at least 5 mm margin of normal-appearing hepatic tissue surrounded the tumor in the hypo-attenuating ablation area. The margins were assessed in the three spatial dimensions by measuring the distance between the border of the treated lesion and the outer border of the ablation zone.

Comparison of ablation zones with cone beam CT and 1-2 mo MDCT: The effectiveness of the technique was evaluated 1-2 mo after treatment with contrast-enhanced MDCT using standard, previously described terminology[19]. Peri-procedural CBCT images were compared to MDCT images. The ablation zone volume was calculated and compared for both sets of images using specific semi-automatic software (Volume Viewer; GE Healthcare, Waukesha, WI, United States).

Radiation dose: Dose area product (DAP) of each 3D acquisition performed on CBCT was initially stored and the mean DAP was calculated. We also recorded the radiation dose of 1-2 mo post-procedural multiphasic MDCT. In order to compare the radiation dose between CBCT and CT, conversion factors were used to obtain effective dose estimation in milliSievert (mSv). For CT, we used the conversion factor of 0.015 mSv/mGy per centimeter which is commonly used. The issue of patient dose in C-arm CBCT is complex. No conversion factor has been yet broadly adopted for 3D acquisitions with CBCT. Researchers estimated a DAP to effective dose coefficient for hepatic interventions of 0.16 mSv/mGy per square centimeter. We extrapolated this result to calculate a mean effective dose.

Statistical analysis

Numerical variables were expressed as medians and ranges. Categorical variables were summarized as numbers and percentages. Ablation zone volumes ob-tained from peri-procedural CBCT and from 1-2 mo post-procedural MDCT were compared using the non-parametric paired Wilcoxon *t*-test ($P < 0.05$ was considered to be significant). Ablation zone volumes measured on peri-procedural CBCT and 1-2 mo post-procedural MDCT were compared with the Bland and Altman plot and Pearson's correlation coefficient. Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, United States).

RESULTS

Ablation procedures

All tumors were treated according to protocol and covered completely as confirmed by ultrasound. Complete ablation was confirmed 1-2 mo post-CBCT on MDCT. Thus the rate of primary effectiveness was 100%. There were no complications related to ablation. Local tumor recurrence and new liver tumors were found 3 mo after initial treatment in one patient (4%).

In this study, the delay between preoperative CT and tumor ablation ranged from 1 to 58 d (median, 7 d) and the delay between tumor ablation and post-operative CT ranged from 28 to 63 d (median, 47 d).

Image quality of cone beam CT

Image quality was poor, good, and excellent in 2 (9%), 8 (35%), and 13 (56%), patients respectively (Table 1). The best image quality was observed in the 20 s group [8/9 excellent examinations (89%) *vs* 5/14 (36%) in the 10 s group, $P = 0.04$]. In the 2 patients with poor quality CBCT images, the ablation volume could not be visualized because of the very peripheral position of the tumor ($n = 1$) and artifacts from significant movement during acquisition ($n = 1$).

Comparison of CBCT with baseline MDCT, and 1-2 mo MDCT

For patients with visible ablation zones (21/23, 91%), the fusion of baseline MDCT and peri-procedural CBCT images showed satisfactory tumor coverage (Figure 2). The minimal margin ablation of 5 mm was achieved in all cases.

Registration quality between peri-procedural CBCT and baseline MDCT images was excellent in 13 (57%) patients, good in 4 (17%) patients, and bad in 6 (26%) patients (Table 1). The median registration time was 5 min (range: 3-8 min). The best registration was observed in the 20 s group [6/9 excellent registrations (67%) *vs* 6/14 (43%) in the 10 s group, $P = 0.04$]. Figure 2 shows a comparison between peri-procedural CBCT and both baseline and 1-2 mo post-procedural MDCT images.

The median ablation zone volumes of peri-procedural CBCT and 1-2 mo post-procedural MDCT images were 30 cm^3 (range: 4-95 cm^3) and 30 cm^3 (range: 4-124 cm^3), respectively (*P*-value > 0.2) (Table 1). There was a good correlation ($r = 0.79$) between the volumes of the two techniques. Figure 3 shows the distribution of the ablation zone volumes assessed by both CBCT and 1-2 mo post-procedural MDCT images, and the Bland-Altman limits of agreement.

Radiation dose

The median DAP of 3D acquisitions performed on CBCT was 43.8 $Gy.cm^2$ (range: 21-74 $Gy.cm^2$). The median effective dose was 7.0 mSv (range: 3.3-11.8 mSv) for CBCT. The median effective dose was 36 mSv (range: 20-52 mSv) for all CT phases and 14.7 mSv (range: 6-27 mSv) for one CT acquisition.

DISCUSSION

This study evaluated a combination of ultrasound and contrast-enhanced CBCT for the assessment of hepatic tumor ablation. Results show that this combination is

Table 1 Patients and ablation characteristics

Patient	Sex	Age (yr)	Tumor size (mm)	Tumor volume (cm^3)	Ablation volume (cm^3)		Registration		CBCT image quality
					CBCT	MDCT	Quality	Time (mo)	
1	M	71	30	13.0	40	32	2	3.9	3
2	M	63	25	8.5	10	12	1	3.3	2
3	M	74	32	16.0	37	55	3	3.4	1
4	M	74	35	22.0	77	69	3	3.3	3
5	M	55	40	36.0	58.5	46	2	6.3	3
6	M	46	15	3.0	12	7.5	3	5	3
7	M	46	30	14.0	38	30	3	8	2
8	M	46	12	2.5	30	32	3	8	2
9	M	68	30	13.5	33	59	3	4.2	2
10	M	65	14	3.0	10	17	1	4	1
11	M	86	8	0.5	58	38	1	7	2
12	F	68	20	5.0	4	13	1	5	2
13	M	60	15	3.5	47	34	1	8	2
14	F	56	30	13.0	32	38	3	7	3
15	F	84	22	6.0	10	12	3	4	3
16	M	54	35	27.0	95	124	3	5	3
17	M	81	24	8.5	8	9	3	5	3
18	F	76	13	3.0	8	6	3	3	3
19	F	76	14	3.5	10	11	2	3	3
20	M	77	18	4.0	31	29	3	5	3
21	M	77	11	2.5	13	30	3	5	3
22	F	50	13	3.0	27	19	2	6	3
23	M	57	14	3.5	7	4	1	8	2

Registration quality of the liver contours was rated as follows: (1) poor registration (> 20 mm for liver contours); (2) good registration (between 5-20 mm for both liver vessels and liver contours); and (3) excellent registration for both liver vessels and liver contours (< 5 mm for both liver vessels and liver contours). Cone beam computed tomography (CBCT) image quality was evaluated as follows: (1) poor quality: no visible ablation; (2) good quality: detection of ablation but presence of artifacts which hamper clear delineation; and (3) excellent quality: detection of ablation and no artifacts. Registration time corresponds to the duration of the software manipulation to achieve the best image fusion between baseline computed tomography and periprocedural CBCT. MDCT: Multidetector computed tomography.

feasible, effective in assessing tumor coverage, and can provide immediate information on the ablation zone, which is well correlated to the reference CT performed one to two months after the procedure.

The goal of monitoring is to identify changes that occur during tumor ablation and determine tumor coverage, which is a key factor in local tumor progression[15]. Ultrasound, which is effective for tumor targeting, is limited for ablation monitoring, mainly due to the production of gas during the procedure. However, during ultrasound guided-ablation, the addition of a contrast agent (CEUS) provides important information for immediate treatment assessment, to detect and remove residual viable tumor areas[20]. The first aim of this study was to determine whether contrast-enhanced CBCT could be a useful tool and an interesting alternative to CEUS or MDCT for assessing the technical success of treatment. The use of intravenous contrast-enhanced acquisitions provided significant attenuation differences between the treated area and the adjacent liver parenchyma. In this study, the reliability of this technique was very good, since the contrast-enhanced CBCT images visualized the targeted area in all patients except two (9%). Moreover, limited results were observed in the first patients and were due to the learning curve.

Protocol optimization including a decrease in the CBCT rotation speed and patient positioning clearly improved the quality of images. Tumor coverage was satisfactory in all cases with at least a 5-mm margin of normal-appearing hepatic tissue around the tumor within the hypo-attenuating ablated area on fusion images. Tumor coverage is even more important in large tumors treated by a multiprobe technique or other tumor ablation techniques requiring careful needle placement. In these cases, immediate visualization of tumor coverage can be used to reposition the needles and perform an additional ablation. In the present series, tumor coverage was satisfactory so that electrode needle replacement was not necessary. Indeed, combining the CBCT technology and integrated navigation software can also help guide needles during percutaneous tumor ablation[16].

The second aim of this study was to determine whether contrast-enhanced CBCT could predict the ablation volume immediately after the ablative procedure. Thus, peri-procedural CBCT images were compared with 1-2 mo post-procedural MDCT used as the reference imaging modality. There was an excellent correlation between ablation zone volumes. Interestingly, besides the quantitative results, image registration of the two approaches matched very well in most cases, confirming that immediate assessment of the ablation zone was highly reliable. These results are similar to those published by Iwazawa *et al*[18] indicating that the combination of ultrasound and peri-procedural CBCT provides an interesting alternative to MDCT. This combination could become the standard of care in centers in which

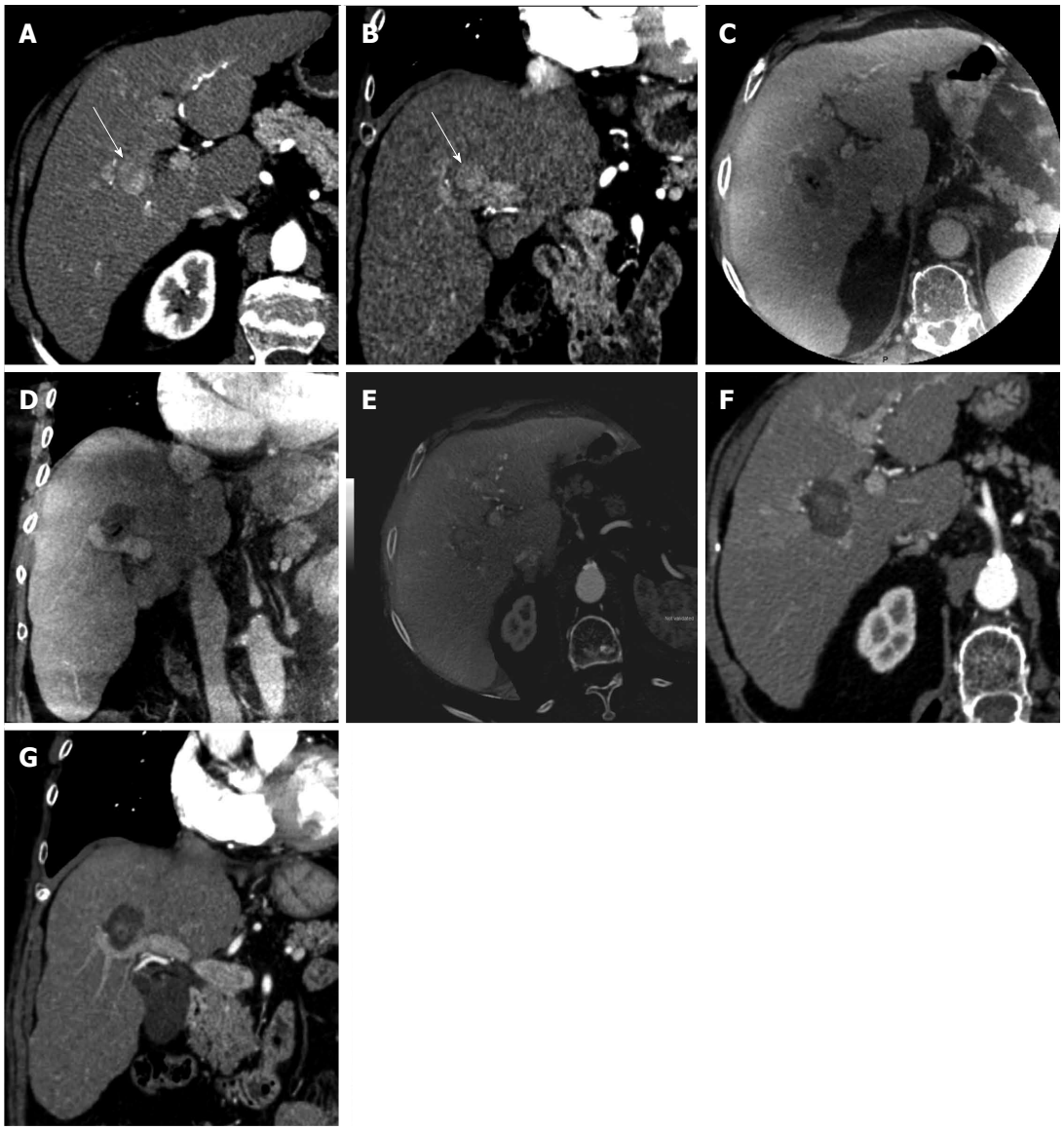

Figure 2 A 53-year-old man with small hepatocellular carcinoma in the right liver. On baseline arterial phase axial and coronal contrast-enhanced multidetector computed tomography (MDCT) (A and B), the tumor was hypervascular (arrow). On axial and coronal contrast-enhanced cone beam computed tomography (CBCT) immediately after radiofrequency ablation (C and D), the ablation zone was clear and markedly hypoattenuating. Image fusion performed between baseline arterial phase contrast-enhanced MDCT and contrast-enhanced CBCT showed satisfactory tumor coverage (E). The ablation zone is clearly visible on 1-mo post-procedural axial and coronal contrast-enhanced MDCT (F and G). The volume of necrosis obtained from contrast-enhanced CBCT and 1-mo post-procedural contrast-enhanced MDCT was similar.

interventional radiology CT is not available. In these centers, the use of CBCT rather than diagnostic CT could be more cost-effective, and make diagnostic CT available to other patients. Further study is needed on this issue.

Indeed, despite the advantages of CBCT in interventional radiology rooms, the quality of images with this technology is not as good as that of conventional MDCT. The increased scattered radiation generated by CBCT results in image artifacts, decreased contrast-to-noise, and inaccuracies in CT calculations[13]. Although this new technique will not replace MDCT to detect local tumor progression in the near future, it can provide peri-procedural information to identify incomplete treatment.

CBCT requires increased doses of radiation. However, this is difficult to evaluate because there is no standard dose metric. For instance, the CT dose index and the dose length product do not correctly apply to cone beam geometries in a flat panel detector. Recently, Strocchi *et al*[21] compared the dose of cone beam CT and MDCT in thoracic biopsy procedures. The authors concluded that although the differences were not always statistically significant, the general distribution of organ doses showed that the MDCT-guided doses were higher than CBCT-guided doses.

We also wanted to compare the radiation dose of CBCT acquisition and CT. This task is rather difficult because there is a lack of a universally accepted common dose metric. For instance, CT dose index and the dose length product do not correctly apply to cone beam geo-

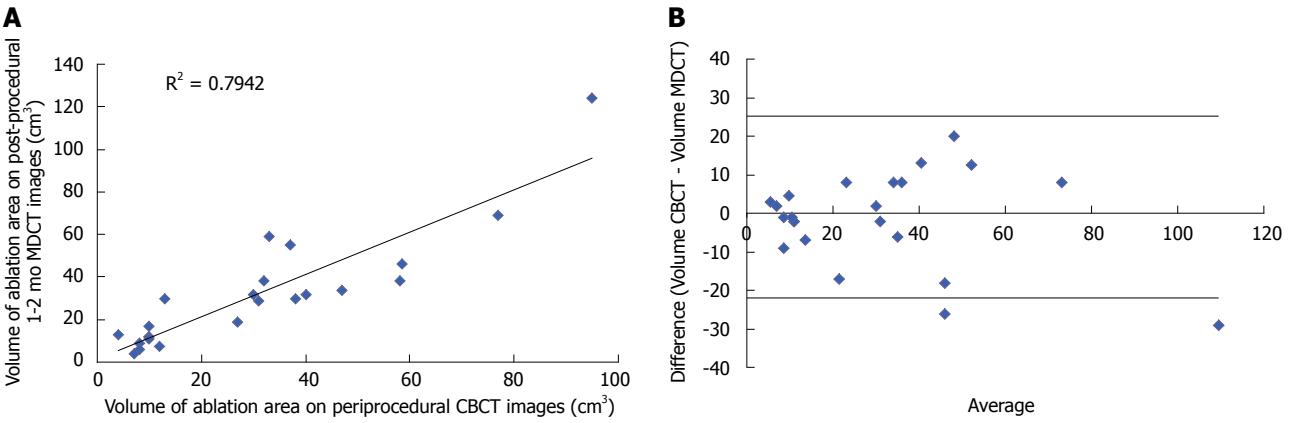

Figure 3 Comparison of cone beam computed tomography with baseline multidetector computed tomography, and 1-2 mo multidetector computed tomography. A: Correlation between ablation zone volumes calculated from cone beam computed tomography (CBCT) images and those from 1-2 mo post-procedural multidetector computed tomography (MDCT) images; B: Bland and Altman limits of agreement for the ablation zone volumes measured on CBCT and MDCT.

metries of a flat panel detector and the dose is non-linear with the central slice getting the highest dose. However imperfect and indirect, we have found that the median effective dose of CBCT acquisition was inferior to that of MDCT acquisition. Our results are in agreement with recent publications[22,23].

Besides its monocentric, retrospective design, the present study has certain limitations. Firstly, CBCT acquisitions were obtained during the portal venous phase to maximize the contrast between the ablation zone and the liver parenchyma. Nevertheless, the value of arterial-phase CBCT acquisitions for detecting residual arterial enhancement was not assessed. Secondly, an acquisition field of view of 40 cm provides a reconstruction field of view of 23.2 cm due to the magnification effect caused by source image distance. Therefore, patient positioning is important, especially in obese patients. Indeed, positioning should be planned before the procedure, so that the ablation zone is in the center of the acquired volume. In addition, the study population was fairly small. Finally, post-procedural MDCT was performed at different times after the ablation (1-2 mo). This might create a potential bias due to the progressive modification of the ablation zone. Nevertheless, a significant correlation was observed between ablation volume on peri-procedural CBCT and post-procedural MDCT. This suggests that these modifications are limited and do not affect assessment of the ablation volume.

In conclusion, this study shows that immediate intravenous contrast-enhanced CBCT following percutaneous tumor ablation of the liver is feasible and could provide early assessment of the ablation zone and margins.

COMMENTS

Background

Ablation is an increasingly used treatment for focal liver lesions, particularly hepatocellular carcinoma and liver metastases. The risk of local tumoral progression after the treatment is strongly associated with the volume of ablation, and especially the presence of peritumoral safety margins. Early assessment of the ablation volume is therefore very important.

Research frontiers

Early evaluation of ablation area can be performed with immediate contrast-enhanced cross-sectional imaging such as computed tomography (CT) or magnetic resonance (MR). However, many teams use ultrasound guidance for liver ablation. In this setting, ablation volume is not easily evaluated. The authors show that contrast-enhanced cone beam computed tomography performed immediately after ablation may help in visualizing of the ablation volume.

Innovations and breakthroughs

Recent studies have demonstrated the clinical value of cone beam computed tomography (CBCT) for the guidance, monitoring and evaluation of numerous vascular and non-vascular interventional radiology procedures such as biopsies or intra-arterial therapies. This study enlarges the field of application of contrast-enhanced CBCT to liver ablation procedures.

Applications

By showing ablation volume immediately after ablation, these results may lead to a more confident ablation procedure, avoiding the use of CT or MR which can therefore be dedicated to diagnostic purposes, and hopefully decrease the rate of local tumoral progression.

Terminology

CBCT refers to a recently developed imaging technique using X-ray. During the image acquisition, an X-ray source rotates around the patient and enables the acquisition of a volume of interest. The technology resembles that of CT, but is performed in interventional radiology rooms using a flat panel detector. It provides three-dimensional rendering of tissues and is very useful for catheter vascular and non-vascular procedure guidance, monitoring and evaluation.

Peer review

The authors examined the feasibility, image quality and immediate interest of contrast-enhanced CBCT for the evaluation of ablation volume after liver ablation. They showed that contrast-enhanced CBCT is feasible and could provide early assessment of the ablation zone and margins. They also showed that CBCT images are well correlated with the reference CT performed one to two months after the procedure. These results are interesting and may indicate that CBCT can provide early additional information for the management of the patients.

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ORIGINAL ARTICLE

Retrospective Study

Patient age and duration of colonoscopy are predictors for adenoma detection in both proximal and distal colon

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Abstract

AIM: To investigate the relation of patient characteristics and procedural parameters to the endoscopic detection rate of colonic adenomas. Further to study, which factors may be capable to predict the localization of adenomatous lesions.

METHODS: We used the data base of a prospective randomized colonoscopy study (The ColoCap trial) to identify patients being diagnosed with colon adenoma. Logistic regression analysis was conducted to reveal predictors for adenoma detection in the entire colon and also with respect to the proximal and distal part. Covariates including age, gender, duration of colonoscopy and comorbidities were defined to determine association between predictors and adenoma detection.

RESULTS: Equal numbers of adenomas were detected in the proximal and distal side of the splenic flexure [126 (57%) *vs* 94 (43%), $P = 0.104$]. Simultaneous occurrence of adenomas in both sides of the colon was rare. The appearance of both proximal and distal adenoma was associated with increasing age ($P = 0.008$ and $P = 0.024$) and increasing duration of colonoscopy ($P < 0.001$ and $P = 0.001$). Male gender was a predictor for adenoma detection in the proximal colon ($P = 0.008$) but statistical significance was slightly missed with respect to the distal colon ($P = 0.089$). Alcohol abuse was found to be a predictor for the detection of distal adenoma ($P = 0.041$).

CONCLUSION: Increasing age and longer duration of colonoscopy are factors with a strong impact on adenoma detection both in the proximal and distal colon. Since proximal adenomas occurred in absence of distal adenomas, complete colonoscopy should be performed for screening.

Key words: Adenoma; Colorectal carcinoma; Distal; Colonoscopy; Proximal

Core tip: In this post-hoc study of a prospective randomized trial we analysed the impact of predefined patients and procedural characteristics on adenoma detection. Proximal lesions are at risk for being missed during colonoscopy but data is sparse regarding the existence of specific predictors for the detection of proximal and distal adenomas. Therefore, in our analysis we computed side specific regression analysis in order to define those predictors. Male gender, longer duration of colonoscopy procedure and increasing age were predictors for both proximal and distal adenomas. Proximal adenomas frequently occurred in the absence of distal adenomatous lesions. We therefore suggest total colonoscopy instead of sigmoidoscopy for colorectal cancer screening.

Klare P, Ascher S, Hapfelmeier A, Wolf P, Beitz A, Schmid RM, von Delius S. Patient age and duration of colonoscopy are predictors for adenoma detection in both proximal and distal colon. *World J Gastroenterol* 2015; 21(2): 525-532 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/525.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.525

INTRODUCTION

The endoscopic detection and resection of adenomatous polyps in the colon is the gold standard for colorectal cancer (CRC) prevention. Many countries have established screening colonoscopy programs, which has lead to a decrease in CRC incidence[1]. However, there is controversial, whether both distal and proximal colonic malignancies can sufficiently be prevented by screening colonoscopy. Lesions located in the cecum, ascending and transverse colon and splenic flexure are usually assigned to the right (or proximal) colon, whereas the left colon includes the descending and sigmoid colon as well as the rectum[2]. Some studies have suggested that benefits from colorectal cancer screenings are stronger for the left than for the right side of the colon[3,4]. This fact has led to a new debate on the significance of flexible sigmoidoscopy as the primary screening method[5,6]. In fact, little is known about the local distribution of adenomas in the large intestine. The adenoma detection rate is an essential colonoscopy quality indicator and is applied for the entire colon[7,8]. So far, it has not been investigated if the acquisition of separate adenoma detection rates for both proximal and distal colon sections may be useful. If the frequency or appearance of proximal adenoma was different from distal lesions, one would also have to ask which factors may influence the respective pattern. Until now, no side-specific risk factors have been established. Only few studies have focused on a possible heterogeneity between proximal and distal adenomatous lesions and emphasized the impact of epidemiological factors on the local distribution[9-11].

The aim of this study therefore was to determine patient characteristics as well as procedural measurements, which affect the detection of proximal and distal colon adenomas. As we conducted a post-hoc analysis of a former patient safety study another focus was placed on sedation related variables.

MATERIALS AND METHODS

We used the database of a large prospective randomized trial (ColoCap Study) which was conducted at three centers in Germany between January 2010 and January 2011[12]. The aim of the ColoCap Study was to determine the value of capnography as a tool for detection and monitoring apnea during colonoscopy under propofol sedation[12]. For a post-hoc analysis pathological data of patients and resected lesions were abstracted by reviewing patient medical records and the institutional electronic charting system.

Procedures were done with standard white light video-colonoscopes. All polyps were resected by forceps biopsy or snare polypectomy according to national guidelines[7] and sent for pathological investigation.

According to histopathological findings lesions were divided in the following categories: no pathology, hyperplastic polyp, adenoma (tubular, villous, serrated) and carcinoma. We defined adenocarcinoma as well as adenoma which presented villous histology as "advanced lesions". An advanced lesion was also registered in case if adenoma were greater than or equal 10 mm in size regardless of further histopathological findings. Lesions were divided into two groups depending on the area of detection. We defined the right colon to begin with the cecum reaching up to (and including) the splenic flexure. All polyps which were found in this part were grouped under the term "right sided" or "proximal lesions". Lesions that were harvested further down were assigned to the left (or distal) colon.

We summarized variables dealing with patient safety and monitoring under the category "procedural characteristics". These included the amount of sedatives used, occurrence of hypoxemia and bradycardia but also duration of colonoscopy. Patient characteristics as well as colonoscopy findings were grouped into two other categories of variables. Variables of all three categories were analyzed in order to reveal possible predictors for the detection of colonic lesions. The post-hoc analysis was approved by the Ethics Committee of the Technical University of Munich (project number: 5793/13).

Statistical analysis

Descriptive statistics of continuous and categorical data are given by mean, range, and absolute and relative frequencies. Uni- and multivariate analysis were performed by logistic regression. In case of semi-complete separation, Firth's penalized-likelihood logistic regression was used for a robust estimation of the odds ratio. 95%CI are presented for the latter. For categorical data the odds ratio describes the ratio of odds of a

Table 1 Patient and procedural characteristics of 551 cases included in the analysis

Patients	
Age (yr)	62 (18-90)
Gender: male/female	285 (52)/266 (48)
Setting: in-patient/out-patient	298 (54)/249 (46)
BMI	24.8 (15.1-41.5)
Sleep apnea syndrome: yes/no	17 (3)/528 (97)
Previously known sedation related complications: no/yes	540 (99)/7 (1)
ASA classification: Ⅰ / Ⅱ / Ⅲ	160 (29)/215 (39)/173 (32)
Lung disease: yes/no	59 (11)/489 (89)
Heart disease: yes/no	132 (24)/416 (76)
Procedural measurements and safety monitoring	
Using capnography	267 (50)
Investigation time (min)	32 (2-126)
Hypoxemia (at least one episode)	241 (44)
Hypoxemia (p02 < 90%; at least one episode)	71 (13)
Hypoxemia (p02 < 85%, at least one episode)	23 (4)
Hypotension (at least one episode)	10 (2)
Bradycardia (at least one episode)	50 (9)
Using midazolam	57 (10)
Using propofol	526 (95)
Propofol dose (mg)	140 (0-800)

Numbers are mean values (range: minimum-maximum) or frequencies (percentages). BMI: Body mass index.

Table 2 Colonoscopy outcome and Procedural findings (n = 551) n (%)

Finding	Total/detection rate
Colonic lesions (entire colon)	
Lesions total (polyps, adenomas, carcinomas)	430
Polyps	412
Adenomas	220
Advanced adenomas	41
Carcinomas	18
Polyp detection rate[1]	37%
Adenoma detection rate[1]	22%
Cases with at least 1 adenoma	121
Cases with 2 or more adenomas	46
Other colonic pathology	
Hemorrhoids	19 (3)
Stenosis	4 (1)
Angiodysplasia	14 (3)
Diverticula	53 (10)
Mucosal Bleeding	5 (1)
Mucosal inflammation	86 (16)

[1]Detection rates were defined as number of colonoscopies in which one or more lesion was found divided by the number of colonoscopies performed.

category and the reference category. In continuous data it describes the ratio of odds of a subject with value x + 1 and a subject with value x. In detail, odds ratio describes the quotient of chances to reveal one characteristic (*e.g.*, "adenoma") dependent whether one predefined factor is present or not. For example, this factor might be patient age. x + 1 would mean to increase age by one unit (year). Likewise, for categorical data the odds ratio describes the ratio of odds of a category and the reference category. Dichotomization of continuous variables was not done due to the potential loss of information. All analyses were performed in an explorative manner on a 5% significance level. IBM SPSS Statistics 20 (SPSS inc., Chicago, IL, United States) and the statistical software package R 2.15.1 (The R Foundation for Statistical Computing, Vienna, Austria) were used for computation.

RESULTS

Patient and procedural characteristics

Complete data were available from 610 in- and outpatient patients. Fifty-nine colonoscopies were excluded because of indications referred to as "polypectomy", "evaluation of known CRC", "inflammatory bowel disease" and "polyposis syndrome". Thus, a total of 551 records were analyzed. Ninety-nine patients (18%) were admitted to colonoscopy for screening. Additionally, 39 (7%) were investigated because of former polypectomy (surveillance). Rectal bleeding or anemia and abdominal discomfort comprised 167 (30%) and 155 (28%) of all cases. Patients had a mean age of 62 years, gender was distributed evenly (285 male, 266 female). Patient characteristics including use of drink and tobacco and medical history are shown in Table 1.

Colonoscopy took at mean 32 min. Almost all patients (526, 95%) received propofol whereas midazolam was administered in only 57 cases (10%). Mean propofol dose was 140 mg per session (range: 0-800 mg). Episodes of hypoxemia (at least one episode of decrease of oxygen saturation $\geqslant$ 5 percentage points or oxygen saturation < 90%) were observed in 241 cases (44%). Frequency was much lower when only the latter definition of hypoxemia (oxygen saturation < 90%) was considered (71 cases, 13%). Patient monitoring data is shown in Table 1.

Pathology

A total of 430 colonic lesions were detected including 220 adenomas. Among them were 41 advanced lesions (19%) including 18 adenocarcinomas. Polyp and adenoma detection rates (defined as numbers of colonoscopies with at least one lesion divided by total amount of records) were 37% and 22%, respectively. More than one adenomatous lesion was found in 8% of all colonoscopies. We found at least one advanced adenoma in 36 (7%) cases.

In 104 of 121 cases (86%) in which adenomas were found, lesions were resected immediately after detection. Resection was performed using biopsy forceps in 53 (44%) and polypectomy snare in 49 cases (40%). In 2 cases no data on the mode of resection was available. In 17 cases (14%) lesions were not resected in the same session when they were detected. Besides polypoid lesions and carcinoma colonoscopies revealed further findings which are listed in Table 2. The most frequent pathology was mucosal inflammation, which was described in 16% of all cases.

Global adenoma detection rate

Univariate analysis: Increasing age and male gender were significantly associated with adenoma detection ($P < 0.001$, OR = 1.026, 95%CI: 1.012-1.1041 and $P = 0.003$, OR = 0.535, 95%CI: 0.353-0.812). The definition of odds ratio is described in the statistical section of "Materials and Methods". Therefore, regarding age, an OR of 1026 can be interpreted as meaning that every additional patient year increased the risk (odds) of detecting at least one adenoma by 2.6%. For the categorical parameter sex the abovementioned odds ratio of 0.535 means that female gender decreased the risk for harvesting adenoma by 53%. Regarding lifestyle both, the use of tobacco and alcohol intake significantly predicted adenoma detection ($P = 0.019$, OR = 0.610, 95%CI: 0.404-0.921 and respectively $P = 0.050$, OR = 0.538, 95%CI: 0.290-1.000). Body composition or comorbidities were not associated with adenoma detection.

Increasing investigation time was a significant factor for detecting adenoma. Every additional minute increased the chance to detect at least one adenomatous polyp by 2.7% ($P < 0.001$, OR = 1.027, 95%CI: 1.018-1.037). Furthermore, patients who had suffered from sedation problems during endoscopic procedures in the past were at higher risk for revealing adenoma ($P = 0.041$, OR = 0.207, 95%CI: 0.046-0.940). The amount of propofol used in one session predicted the detection of adenomas but missed statistical significance slightly. Indication for colonoscopy was a predictor for adenoma detection ($P < 0.001$). Odds ratio for the indication "suspected tumor" was 3.676 (95%CI: 1.118-12.018). Other procedural characteristics like colonoscopy time schedule (earlier/later in the day) or sedation related complications (hypoxemia, hypotension and others) had no impact on adenoma detection.

The occurrence of inflammation (at least one inflamed area described during colonoscopy) was significantly associated with lower adenoma detection ($P < 0.001$, OR = 9.408, 95%CI: 2.918-30.333).

Multivariate analysis: Regarding the whole colon both increasing age and male gender were stable variables to predict adenoma detection ($P = 0.002$, OR = 1.032, 95%CI: 1.012-1.053 and $P = 0.007$, OR = 0.507, 95%CI: 0.309-0.832). In contrast, lifestyle factors (drink and tobacco) were no longer significant predictors after controlling for confounders.

Longer investigation time remained significantly associated with increased detection of adenomatous lesion in multivariate analysis ($P < 0.001$, OR = 1.033, 95%CI: 1.019-1.048). Indication for colonoscopy also predicted adenoma detection ($P < 0.001$). Odds ratio for the indication "suspected tumor" was 3.399 (95%CI: 0.859-12.914). All other procedural and safety measurements failed statistical significance. Similarly, mucosal inflammation as an endoscopic finding was no longer evident after adjusting for confounders by regression analysis.

Advanced lesions

We found 41 advanced adenomas. Among these, 18 lesions were classified as adenocarcinomas. Advanced adenoma detection rate was 7%. In univariate analysis increasing age ($P = 0.004$, OR = 1.040, 95%CI: 1.013-1.067), male gender ($P = 0.014$, OR = 0.389, 95%CI: 0.184-0.823), longer duration of colonoscopy ($P < 0.001$, OR = 1.032, 95%CI: 1.018-1.045), inpatient setting ($P = 0.001$, OR = 0.190, 95%CI: 0.072-0.499), colonoscopy performed later in the day ($P < 0.001$, OR = 1.412, 95%CI: 1.189-1.678) and higher propofol doses ($P = 0.024$, OR = 1.003, 95%CI: 1.000-1.005) were predictors for the detection of advanced lesions. Every additional hour that endoscopy started later, the chance to detect an advanced lesion rose by 40%. Indication for colonoscopy was also a predictor for the detection of advanced adenomas ($P < 0.001$). Odds for the indication "suspected tumor" was 6.942 (95%CI: 0.869-55.480). No multivariate regression analysis was performed in this sub-setting of the analysis due to the rarity of the outcome.

Serrated adenoma

Pathologic investigation revealed a serrated phenotype in six out of 220 adenomas. Five of these six serrated lesions were located in the distal colon. Only one serrated adenoma (SA) located in the distal colon was assessed as an advanced lesion. Predictors for SA detection was indication for colonoscopy [(anemia/bleeding), $P = 0.002$, OR = 0.063, 95%CI: 0.000-0.603] and higher propofol dose ($P = 0.037$, OR = 1.004, 95%CI: 1.000-1.009). Multivariate regression analysis was again not performed in this sub-setting due to the rarity of the outcome.

Comparing lesions in the proximal and distal colon

Side-specific adenoma detection rates: The total count of adenoma was not significantly different in the right and in the left colon [126 (57%) *vs* 94 (43%), $P = 0.104$]. Twenty-three proximal and eighteen distal adenomas were classified as advanced lesions. The local distribution of carcinomas was equal between both sides (9 *vs* 9). Adenoma detection rates were similar in the proximal and distal colon (13% respectively). Furthermore we found no difference with respect to the detection of advanced lesions (3% both). Simultaneous occurrence of adenoma in both parts of the colon was noticed in only 4% (22/551) of all cases. In no case advanced lesions were found simultaneously on both sides of the colon. Descriptive data regarding site-specific detection rates of colon lesions is given in Table 3.

Predictors for proximal adenoma detection: In the proximal colon increasing age ($P < 0.001$, OR = 1.034, 95%CI: 1.015-1.053), male gender ($P = 0.002$, OR = 0.434, 95%CI: 0.255-0.741), increasing duration of colonoscopy ($P < 0.001$, OR = 1.030, 95%CI: 1.019-1.041), indication for procedure [$P < 0.001$, OR = 1.949 ("Suspected tumor"), 95%CI: 0.510-7.445], tobacco abuse ($P = 0.005$, OR = 0.470, 95%CI: 0.279-0.792)

Table 3 Local distribution of colon adenomas in 551 cases *n* (%)

Finding	Total/detection rate
Adenomatous lesion in the right colon	
Adenomas	126
Adenoma detection rate[1]	13%
Advanced adenomas	23
Advanced adenoma deletion rate	19 (3)
Adenomatous lesion in the left colon	
Adenomas	94
Adenoma detection rate[1]	13%
Advanced adenomas	18
Advanced adenoma deletion rate	17 (3)
Simultaneous detection of lesions	
(Cases with at least one adenoma in both right and left colon)	
Adenomas	22 (4)
Advanced adenomas	0

[1]Detection rates were defined as number of colonoscopies in which one or more lesion was found divided by the number of colonoscopies performed.

and mucosal inflammation (P = 0.010, OR = 4.763, 95%CI: 1.463-15.504) predicted adenoma detection in univariate analysis. However, only age (P = 0.008, OR = 1.036, 95%CI: 1.010-1.064), gender (P = 0.008, OR = 0.425, 95%CI: 0.227-0.797) and duration of endoscopy (P < 0.001, OR = 1.039, 95%CI: 1.022-1.056) remained significant factors after controlling for confounders.

Predictors for distal adenoma detection: In the distal colon increasing age (P = 0.024, OR = 1.029, 95%CI: 1.004-1.054) and increasing investigation time (P = 0.001, OR = 1.027, 95%CI: 1.011-1.044) were also associated with improved adenoma detection in the multivariate setting. Male gender was a significant factor in the univariate analysis (P = 0.026, OR = 0.553, 95%CI: 0.329-0.931) but missed the level of statistical significance slightly after controlling for confounders (P = 0.089). Alcohol intake was a predictor for adenoma detection (P = 0.041, OR = 0.438, 95%CI: 0.198-0.967). After adjusting for confounders, patients rated as alcohol abusers were at a 44% higher risk for revealing adenoma compared to abstainers. Regarding procedural measurements a history of previously sedation problems (P = 0.032, OR = 0.190, 95%CI: 0.042-0.866), increasing propofol dose (P = 0.011, OR = 1.002, 95%CI: 1.001-1.004) and indication for colonoscopy [P < 0.001, OR = 7.560 ("suspected tumor"), 95%CI: 0.947-60.343] predicted adenoma detection in the left colon. However, only indication for colonoscopy (P = 0.013) was statistically significant in multivariate analysis [OD = 6.599 ("suspected tumor"), 95%CI: 0.756-75.586]. Two colonoscopy findings (absence of diverticula and mucosal inflammation) were significant factors in univariate analysis (P = 0.037, OR = 8.403, 95%CI: 1.143-61.779 and P = 0.035, OR = 0.032, 95%CI: 0.001-0.227). As these two variables were not predefined as relevant confounders, they were not subject to multivariate testing.

DISCUSSION

The adenoma detection rate is an established quality indicator for colonoscopy[13]. A total detection rate of 20% has been defined as a landmark in screening colonoscopy[14,15]. Adenomatous lesions can occur both in the proximal and distal part of the colon but information is sparse regarding the local distribution[16]. In the past the focus was placed on distal malignancies[17] with flexible sigmoidoscopy propagated as a sufficient tool for detection and surveillance. Advocators of flexible sigmoidoscopy argue that screening colonoscopy has not met expectations insofar as proximal cancer has not been prevented sufficiently[3,4,18,19]. Moreover, sigmoidoscopy has been shown to be effective in reducing cancer in several countries[20-22]. However, there are also some hints that premalignant polypoid lesions might be missed on the left side[23,24]. Little is known about possible risk factors for proximal or distal adenomas respectively.

In this study we sought to describe characteristics of adenomatous lesions separated by the splenic flexure and to reveal factors which affect side-specific adenoma detection. As we considered data from a former patient safety study another focus laid on sedation-related and procedural measurements as well as on available patient characteristics.

Simultaneous occurrence of proximal and distal adenoma is rare

We found a total of 220 adenomatous lesions and determined a total adenoma detection rate of 22%. As the main finding of this study, the amount of adenoma harvested from the proximal colon did not differ significantly from the count harvested from the distal part (57% *vs* 43%). Furthermore, advanced lesions and carcinomas were found with an almost similar distribution on both sides. In a recent study Boroff *et al*[16] found a significantly higher adenoma detection rate in the right colon. Diminutive proximal adenomas are of special interest since a remarkable miss-rate is suspected regarding these lesions[25]. Worthy to note, serrated adenomas, especially the sessile serrated subtype, may present as such small and difficult to detect polyps[26]. Some data suggest that missing these premalignancies may contribute to an increasing risk of developing proximal colon cancer[27,28]. In our investigation six out of 220 adenomas (3%) were serrated lesions. This quota is in agreement with the range reported in the literature[29]. Contrary to the knowledge that serrated lesions frequently occur in the proximal part of the colon, our results revealed a surplus of serrated adenomas in the distal part. This finding might be due to the low number of serrated lesions detected in our study.

Most importantly, simultaneous occurrence of any kind of adenomas in both the proximal and distal colon was rare. Moreover, we did not detect advanced lesions on both sides of the splenic flexure in any patient. This finding underlines the importance of screening

the whole colon instead of performing sigmoidoscopy. Inspecting only the distal part of the colon would mean to accept the risk of missing a relevant number of cancer precursors in the right colon.

Male gender and advanced age are risk factors for both proximal and distal adenomas

We found that older patients and males were exposed to increased risk for revealing adenoma. This relation is already well known with regard for the whole colon[7,8]. Although male gender did not reach statistical significance in multivariate analysis for the left colon still a clear trend was obvious. Therefore, age and gender seem to influence the probability of revealing adenoma similarly in both the proximal and distal colon. Furthermore, in univariate analysis we found smoking to be associated with the occurrence of proximal colon adenoma. Coincidence between use of tobacco and colorectal findings has been studied repeatedly and in most cases smoking was assessed to be a predictor for premalignant neoplasia or carcinoma[30-32]. In a recent study smoking was deemed to bear the risk for proximal CRC[33]. These data are supported by our findings. In contrast there are also publications in which smoking was rather suggested to promote distal lesions[11]. Concerning alcohol consumption we found drinker to be at a 40% higher risk of revealing distal adenomas than abstainers. This influence was stable in both uni- and multivariate analysis. Alcohol intake as a risk factor for colorectal neoplasia was studied before. In most of these trials the effect of alcohol was week or only observed in subgroups[30]. Regarding adenoma locality one study showed that regular intake of spirit drinks was associated with left sided adenoma[34]. However, no increased risk (nor for proximal neither for distal adenoma) was observed in a newer case-control study containing 628 adenoma cases[11]. In summary, data seems to be inconsistent and sparse. Further studies should be conducted to specify the relevance of alcohol drinking in colorectal (pre)neoplastic lesions.

Procedural measurements: Duration of colonoscopy has impact on adenoma detection

At present, a withdrawal time of at least 6 min is classified as a quality indicator in colonoscopy[7,35]. Due to the retrospective study design we were not able to measure the withdrawal time and no data was available regarding the length of mucosal observation in each particular colon segment. In addition, one major limitation of this analysis derives from the fact that we were not able to subtract expenditure of time which was needed for the conduction of polypectomy itself. This fact may have resulted in a bias. In 14% of all cases in which adenomas were found polypectomy was not conducted during the same session and in further 44% resection was carried out immediately using the biopsy forceps which suggests that in the majority of cases bias might have been rather low. Anyway, due to the retrospective design of our study the data is not capable to prove unambiguously that observation time and not polypectomy itself explain our results. Interestingly, duration of procedure had a strong impact on both proximal and distal adenoma detection. To investigate whether independent observation times may lead to differences in segment-specific adenoma detection further studies should be conducted.

As expected, indication for colonoscopy was a predictor for adenoma detection in our study. As odds were high in cases where tumors were suspected prior to the investigation this finding is comprehensible and noncritical. Regarding the safety of colonoscopy we could not find an association between sedation problems such as hypoxemia or hypotension and a lower adenoma detection rate. However, some other sedation-related measurements were noticeable. We found that a history of former sedation-related complications as well as increasing dose of propofol were predictors of increased adenoma detection in the distal colon but not in the proximal part. The required amount of propofol varies highly between patients and in part depends on age, indication of colonoscopy and physical condition[36-38]. It might be argued that age and comorbidities affect the occurrence of adenoma as well as sedation problems. Until now, no sedation-induced effect on adenoma detection has been verified[39].

Limitations

Our study is subject to some limitations. First, since we conducted a post hoc analysis of a former colonoscopy study the retrospective view implies a major restriction. Second, due to the rarity of advanced and serrated adenomas only univariate analysis was performed with respect to these lesions. These results should be interpreted with caution as possible confounders might not be eliminated. In particular, predictors for advanced lesions like inpatient setting and colonoscopy scheduled later in the day must be interpreted with caution since inpatients are investigated later in the day for organizational reasons. Third, in our setting we were faced with a mixed patient population undergoing colonoscopy for a multitude of indications which prevents transferring the results to a cancer screening scenario and procedures were conducted at a single tertiary referral center which might explain the high proportion of advanced lesions and carcinoma that were detected. Finally, in our data we were not able to provide information about quality of bowel preparation, a factor which influences adenoma detection.

In summary, our data support the assumption that male gender, advanced age and a longer duration of colonoscopy are related with increasing rates of adenoma detection. This finding applies to both proximal and distal adenomatous lesions. We found no evidence that sedation related complications influence adenoma detection. Furthermore, our data reveal that proximal lesions often occur in the absence of distal adenomas. Therefore, total colonoscopy should be preferred to

sigmoidoscopy in case of colorectal cancer screening.

COMMENTS

Background

To determine predictors for the detection of proximal and distal adenomas.

Research frontiers

Adenomas in the proximal colon are at risk of being missed during screening colonoscopy.

Innovations and breakthroughs

Patient age, male gender and duration of colonoscopy were predictors for both proximal and distal adenoma detection. Proximal adenomas were detected frequently in absence of distal adenomatous lesions.

Applications

Full colonoscopy should be performed instead of sigmoidoscopy for the detection of cancer precursors.

Terminology

Adenomatous polyps detected in the cecum, ascending or transverse colon are defined as "proximal adenomas".

Peer review

This paper is an interesting study on the factors influencing the adenoma detection rate in colonoscopy. The authors use a colonoscopy database designed for other aims which make it easy to avoid bias in patient selection.

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ORIGINAL ARTICLE

Retrospective Study

Thrombomodulin in the management of acute cholangitis-induced disseminated intravascular coagulation

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Abstract

AIM: To evaluate the need for thrombomodulin (rTM) therapy for disseminated intravascular coagulation (DIC) in patients with acute cholangitis (AC)-induced DIC.

METHODS: Sixty-six patients who were diagnosed with AC-induced DIC and who were treated at our hospital were enrolled in this study. The diagnoses of AC and DIC were made based on the 2013 Tokyo Guidelines and the DIC diagnostic criteria as defined by the Japanese Association for Acute Medicine, respectively. Thirty consecutive patients who were treated with rTM between April 2010 and September 2013 (rTM group) were compared to 36 patients who were treated without rTM (before the introduction of rTM therapy at our hospital) between January 2005 and January 2010 (control group). The two groups were compared in terms of patient characteristics at the time of DIC diagnosis (including age, sex, primary disease, severity of cholangitis, DIC score, biliary drainage, and anti-DIC drugs), the DIC resolution rate, DIC score, the systemic inflammatory response syndrome (SIRS) score, hematological values, and outcomes. Using logistic regression analysis based on multivariate analyses, we also examined factors that contributed to persistent DIC.

RESULTS: There were no differences between the rTM group and the control group in terms of the patients' backgrounds other than administration. DIC resolution rates on day 9 were higher in the rTM group than in the control group (83.3% *vs* 52.8%, $P < 0.01$). The mean DIC scores on day 7 were lower in the rTM group than in the control group (2.1 ± 2.1 *vs* 3.5 ± 2.3, $P = 0.02$). The mean SIRS scores on day 3 were significantly lower in the rTM group than in the control group (1.1 ± 1.1 *vs* 1.8 ± 1.1, $P = 0.03$). Mortality on day 28 was 13.3% in the rTM group and 27.8% in the control group; these rates were not significantly different ($P = 0.26$). Multivariate analysis identified only the absence of biliary drainage as significantly associated with persistent DIC ($P < 0.01$, OR = 12, 95%CI: 2.3-60). Although the difference did not reach statistical significance, primary diseases (malignancies) ($P = 0.055$, OR = 3.9, 95%CI: 0.97-16) and the non-

use of rTM had a tendency to be associated with persistent DIC (P = 0.08, OR = 4.3, 95%CI: 0.84-22).

CONCLUSION: The add-on effects of rTM are anticipated in the treatment of AC-induced DIC, although biliary drainage for AC remains crucial.

Key words: Disseminated intravascular coagulation; Acute cholangitis; Thrombomodulin; Biliary drainage; Anticoagulant therapy

Core tip: To evaluate the need for thrombomodulin (rTM) in the management of acute cholangitis (AC)-induced disseminated intravascular coagulation (DIC), we retrospectively compared patients treated with rTM (rTM group) and without rTM (control group). DIC resolution rates were higher in the rTM group (P < 0.01). Multivariate analysis identified only the absence of biliary drainage as significantly associated with persistent DIC (P < 0.01), while there was a trend towards an association between persistent DIC and a lack of rTM (P = 0.08). Therefore, the add-on effects of rTM are anticipated in the treatment of AC-induced DIC, although biliary drainage remains crucial.

Suetani K, Okuse C, Nakahara K, Michikawa Y, Noguchi Y, Suzuki M, Morita R, Sato N, Kato M, Itoh F. Thrombomodulin in the management of acute cholangitis-induced disseminated intravascular coagulation. *World J Gastroenterol* 2015; 21(2): 533-540 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/533.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.533

INTRODUCTION

In recent years, there have been several reports on the efficacy of recombinant human soluble thrombomodulin (rTM) for the treatment of disseminated intravascular coagulation (DIC) associated with infection[1-4]. Various disorders that cause infections were described in these reports, but none of the studies focused on a single disease. Treatment of the primary disease causing DIC remains the most important factor in the resolution of the pathological conditions that underlie infectious DIC[5], and the prognosis of patients with DIC may be markedly affected by the outcome of treatment of the primary disease. Thus, it is crucial to focus on the primary disease to accurately assess the treatment outcomes of patients with infectious DIC.

In acute cholangitis (AC)-induced DIC, the treatment for AC, including biliary drainage, can immediate resolve DIC. However, some patients still have poor outcomes, and further improvements in therapy are needed. The utility of rTM for the treatment of DIC remains unclear. Our PubMed search on rTM therapy for AC-induced DIC, using terms such as "disseminated intravascular coagulation", "acute cholangitis", and "thrombomodulin", yielded only a single-arm case series that we previously reported[6]. We had reported favorable outcomes in patients who received a therapeutic regimen of rTM for AC-induced DIC. However, the prior series had a small sample size; in this study, we therefore compared a larger group of patients who were treated with and without rTM to evaluate the role of anti-DIC therapy with rTM for AC-induced DIC. This is the first comparative study of rTM in the treatment of AC-induced DIC.

MATERIALS AND METHODS

Patients

Thirty consecutive patients who were diagnosed as having AC-induced DIC and who were treated with rTM at St. Marianna University School of Medicine Hospital between April 2010 and September 2013 were enrolled in this study (rTM group). They were compared to 36 patients with AC-induced DIC who were treated without rTM (before the introduction of rTM therapy at our hospital) between January 2005 and January 2010. Detailed data were available from medical records, which allowed these 36 patients to serve as historical controls for the analysis (control group).

The rTM group included 22 men and 8 women with a mean age ± SD of 77.0 ± 7.7 years. AC was diagnosed and graded according to the 2013 Tokyo Guidelines[7] for the management of AC. AC was severe in 28 patients and moderate in 2 patients, while no patients had mild AC. The primary diseases causing AC were choledocholithiasis in 20 patients, malignant biliary stricture in 9 (pancreatic carcinoma in 5 patients, cholangiocarcinoma in 2, lymph node metastasis of gastric cancer in 1, and malignant lymphoma in 1), and primary sclerosing cholangitis in 1. Based on the DIC diagnostic criteria defined by the Japanese Association for Acute Medicine[8] (Table 1), DIC was diagnosed when the DIC score was 4 or above. The mean DIC score ± SD at the time of DIC diagnosis was 5.4 ± 1.4. The dose of rTM was 380 units/kg per day in 26 patients, while 4 patients received rTM at a reduced dose of 130 units/kg per day, due to renal dysfunction. The duration of rTM treatment was 6 d in all patients. Other anti-DIC drugs used (besides TM) were antithrombin (AT) in 26 patients, gabexate mesilate (GM) in 14 patients, and nafamostat mesilate (NM) in 4 patients (including duplicate counts). The antibiotics used were meropenem (MEPM) in 19 patients, sulbactam/cefoperazone (CPZ/SBT) in 5 patients, doripenem in 5 patients, and tazobactam/piperacilin (TAZ/PIPC) in 1 patient. Biliary drainage was performed in 25 patients but not in 5 patients. Of the patients who did not undergo biliary drainage, 4 patients did not consent, and the presence of cholangitis after the clearance of bile duct stones precluded this procedure in 1 patient.

Table 1 Diagnostic criteria for disseminated intravascular coagulation as defined by the Japanese Association for Acute Medicine

	Score
Systemic inflammatory response syndrome criteria[1]	
≥ 3	1
0-2	0
Platelet count (× 10^3/L)	
< 80 or > 50% decrease within 24 h	3
≥ 80 and < 120; or > 30% decrease within 24 h	1
≥ 120	0
Prothrombin time (Value of patient/Normal value)	
≥ 1.2	1
< 1.2	0
Fibrin/fibrinogen degradation products (mg/L)	
≥ 25	3
≥ 10 and < 25	1
< 10	0
Diagnosis	
≥ 4 points	DIC

[1]Systemic inflammatory response syndrome criteria: Fever of more than 38 ℃ or less than 36 ℃; Heart rate of more than 90 beats per min; Respiratory rate of more than 20 breaths per minute or a $PaCO_2$ level of less than 32 mmHg; Abnormal white blood cell count (> 12000/μL or < 4000/μL or > 10% bands). DIC: Disseminated intravascular coagulation.

The control group included 21 men and 15 women with a mean age ± SD of 75.7 ± 9.4 years. AC was severe in 32 patients and moderate in 4 patients, while no patients had mild AC. The primary diseases causing AC were choledocholithiasis in 19 patients, malignant biliary stricture in 15 patients (pancreatic carcinoma in 6, cholangiocarcinoma in 5, gallbladder cancer in 2, and hepatocellular carcinoma in 2), bilio-jejunal anastomotic stricture in 1, and bile duct stricture due to a hepatic cyst in 1 patient. The mean DIC score ± SD at the time of DIC diagnosis was 5.2 ± 1.2. The anti-DIC drugs used were GM in 30 patients, NM in 18, AT in 16, and danaparoid sodium (DS) in 6 (including duplicate counts). The antibiotics used were MEPM in 14 patients, SBT/CPZ in 14, imipenem/cilastatin in 7, and TAZ/PIPC in 1. Biliary drainage was performed in 24 patients.

Measurements

The rTM group of 30 patients and the control group of 36 patients were compared in terms of patient characteristics [including age, sex, primary disease (malignant/benign)], severity of cholangitis at the time of diagnosis, DIC score at the time of diagnosis, proportion of patients undergoing biliary drainage, and anti-DIC drugs, the DIC resolution rate, the DIC score, the systemic inflammatory response syndrome (SIRS) score, hematological values [platelet count (Plt), fibrin/fibrinogen degradation products (FDP), prothrombin time-international normalized ratio (PT-INR), fibrinogen (Fib), C-reactive protein (CRP), total bilirubin (T-bil)], and treatment outcomes. The day of DIC diagnosis and treatment initiation was designated as day 1, and hematological values were assessed on days 1, 3, 5, 7, and 9. Moreover, DIC resolution was defined as a decrease in the DIC score to 3 or less. The DIC and SIRS scores were expressed as mean ± SD, and hematological data were expressed as median values (quartiles).

A multinomial logistic regression analysis based on the univariate and multivariate analyses was used to identify factors that contributed to the failure of DIC resolution in patients with AC-induced DIC.

Written informed consent was obtained from all patients. This study was approved by the ethics committee of our hospital.

Statistical analysis

Statistical analyses were performed using the χ^2 test, Fisher's exact test, Welch's t test, the Mann-Whitney U test or the Wilcoxon single rank test, as appropriate. Variables that were found to have a potentially significant association with persistent DIC ($P < 0.2$) by univariate analysis were selected for entry into a multiple logistic regression model. P values < 0.05 were regarded as statistically significant. Statistical analyses were performed using the Prism 5 program (Graph Pad Software, Inc., CA, United States) and SPSS (version 19; SPSS, Chicago, IL, United States).

RESULTS

Patient characteristics

There were no significant differences between the rTM group and the control group with respect to age, sex, primary disease, severity of cholangitis, DIC score, SIRS score, or the proportion of patients who underwent biliary drainage at the time of DIC diagnosis. With regards to anti-DIC agents other than rTM that were used, the proportion of patients who received AT was significantly higher in the rTM group, while a higher proportion of patients in the control group received GM, NM and DS were higher (Table 2).

DIC resolution rate

The DIC resolution rate on day 9 was 83.3% (25/30) in the rTM group and 52.8% (19/36) in the control group (significantly higher in the rTM group; $P = 0.009$). The DIC resolution rates on day 7 were 76.7% (23/30) and 50.0% (18/36), respectively, and again, were significantly higher in the rTM group ($P = 0.041$).

DIC scores

Both the rTM and control groups showed a significant decrease in DIC scores from day 3 onward, compared to those on day 1. The comparison between the rTM and control groups revealed no difference in the mean DIC scores at the time of diagnosis, which were 5.4 ± 1.4 in the rTM group and 5.2 ± 1.2 in the control group ($P = 0.524$). However, the mean DIC scores on day 7 were 2.1 ± 2.1 and 3.5 ± 2.3 ($P = 0.018$), and the mean DIC scores on day 9 were 1.8 ± 1.9 and 3.3 ± 2.4, respectively ($P = 0.009$). The mean DIC scores on days 7 and 9 were

Table 2 Comparison of patient characteristics between the recombinant human soluble thrombomodulin and control groups

	rTM group (n = 30)	Control group (n = 36)	P value
Age (yr)	77.0 ± 7.7	75.7 ± 9.4	0.554
Sex (Male/Female)	22/8	21/15	0.203
Primary disease (Benign/Malignant)	21/9	21/15	0.327
Severity of cholangitis (Severe/Moderate)	28/2	32/4	0.845
DIC score	5.4 ± 1.4	5.2 ± 1.2	0.523
SIRS score	2.4 ± 1.3	2.6 ± 1.0	0.599
Biliary drainage	25	24	0.123
Anticoagulant drug			
AT	26	16	< 0.001
GM	14	30	0.002
NM	4	18	0.004
DS	0	6	0.019
Antibiotics			
MEPM	19	14	0.048
IPM/CS	0	7	0.031
DRPM	5	0	0.037
SBT/CPZ	5	14	0.047
TAZ/PIPC	1	1	0.556

DIC: Disseminated intravascular coagulation; SIRS: Systemic inflammatory response syndrome; rTM: Recombinant human soluble thrombomodulin; AT: Antithrombin; GM: Gabexate mesilate; NM: Nafamostat mesilate; DS: Danaparoid sodium; MEPN: Meropenem; IPM/CS: Imipenem/Cilastatin; DRPM: Doripenem; SBT/CPZ: Sulbactam/Cefoperazone; TAZ/PIPC: Tazobactam/Piperacilin.

significantly lower in the rTM group (Figure 1A).

SIRS scores

Compared to day 1, both the rTM and control groups showed a significant decrease in SIRS scores from day 3 onward. There were no differences between the rTM and control groups in terms of the mean SIRS scores at the time of diagnosis, which were 2.4 ± 1.3 in the rTM group and 2.6 ± 1.0 in the control group (P = 0.599). However, the scores on day 3 were 1.1 ± 1.1 and 1.8 ± 1.1 (P = 0.027), respectively, and were significantly lower in the rTM group. Subsequently, the mean SIRS scores in the rTM group remained significantly lower (Figure 1B).

Hematological values

The median hematological values (day 1/day 9) in the rTM group were as follows: Plt, 70.5 (58.8-94.0)/182.0 (80.5-266.5) × 10^3/μL (P < 0.001); FDP, 20.8 (10.8-43.2)/8.8 (5.9-17.9) μg/mL (P = 0.010); PT-INR, 1.27 (1.21-1.52)/1.18 (1.14-1.24) (P = 0.024); Fib, 293.5 (203.5-449.3)/373.0 (284.8-452.3) mg/dL (P = 0.092); CRP, 8.9 (5.9-15.1)/3.6 (2.0-7.8) mg/dL (P < 0.001); and T-bil, 3.8 (1.8-5.4)/1.9 (1.2-3.1) mg/dL (P = 0.023). The Plt, FDP, PT-INR, CRP, and T-bil values on day 9 showed significant improvement compared to those on day 1. In contrast, the median hematological values (day 1/day 9) in the control group were as follows: Plt, 88.5 (70.3-134.5)/155.0 (73.3-249.0) × 10^3/μL (P = 0.024); FDP, 35.4 (14.0-51.5)/21.0 (11.2-36.5) μg/mL (P = 0.155); PT-INR, 1.34 (1.24-1.67)/1.21 (1.08-1.47) (P = 0.054); Fib, 399.0 (243.0-464.0)/302.0 (219.0-445.5) mg/dL (P = 0.180); CRP, 13.6 (9.8-18.2)/4.8 (2.1-8.1) mg/dL (P < 0.001); and T-bil, 4.0 (1.9-6.7)/1.7 (1.1-4.7) mg/dL (P = 0.021). The Plt, CRP and T-bil values on day 9 showed significant improvement compared to the Day 1 values. A comparison of the median hematological values between the rTM and control groups showed that, although the levels of Plt on day 1 were significantly lower in the rTM group (P = 0.023), the levels of Plt on day 9 were higher in the rTM group; this difference did not reach statistical significance (P = 0.699). Although there was no difference in FDP on day 1 (P = 0.157) between the two groups, from day 3 onward (P = 0.045), the level of FDP was significantly lower in the rTM group. The fluctuations in median hematological values are shown in Figure 1C.

Outcomes

The mortality rate on day 28 was 13.3% (4/30) in the rTM group and 27.8% (10/36) in the control group; although mortality was higher in the control group, the difference did not reach statistical significance (P = 0.260). In the rTM group, all 4 deaths were classified as due to malignant tumors. Of the 10 deceased patients in the control group, cancer deaths occurred in 7 patients, and deaths due to worsening DIC were observed in the remaining 3 patients.

Factors contributing to the failure of DIC resolution

The univariate analysis identified primary disease (malignancy) (P = 0.003, OR = 5.3, 95%CI: 1.8-16), absence of biliary drainage (P < 0.001, OR = 16, 95%CI: 3.9-66), non-use of rTM (P = 0.010, OR = 4.5, 95%CI: 1.5-14), and non-use of NM (P = 0.016, OR = 0.26, 95%CI: 0.088-0.76) as factors that significantly contributed to persistent DIC (Table 3). A multivariate analysis was performed, incorporating the factors that were identified by univariate analysis, as well as the non-use of GM (P = 0.107) and Fib < 200 mg/dL (P = 0.186), both of which were factors with P values < 0.2 in the univariate analysis; the absence of biliary drainage (P = 0.003, OR = 12, 95%CI: 2.3-60) was the only factor that was found to contribute to persistent DIC (Table 4). Although the difference did not reach statistical significance, it was observed that primary disease (malignancies) (P = 0.055, OR = 3.9, 95%CI: 0.97-16) and non-use of rTM (P = 0.080, OR = 4.3, 95%CI: 0.84-22) tended to be associated with persistent DIC.

DISCUSSION

Since May 2008, rTM has been available in Japan as a novel therapeutic agent for DIC. In recent years, there have been several reports on the efficacy of rTM, which binds to thrombin and activates protein C to exert an anticoagulant effect[9,10], for the treatment of infectious

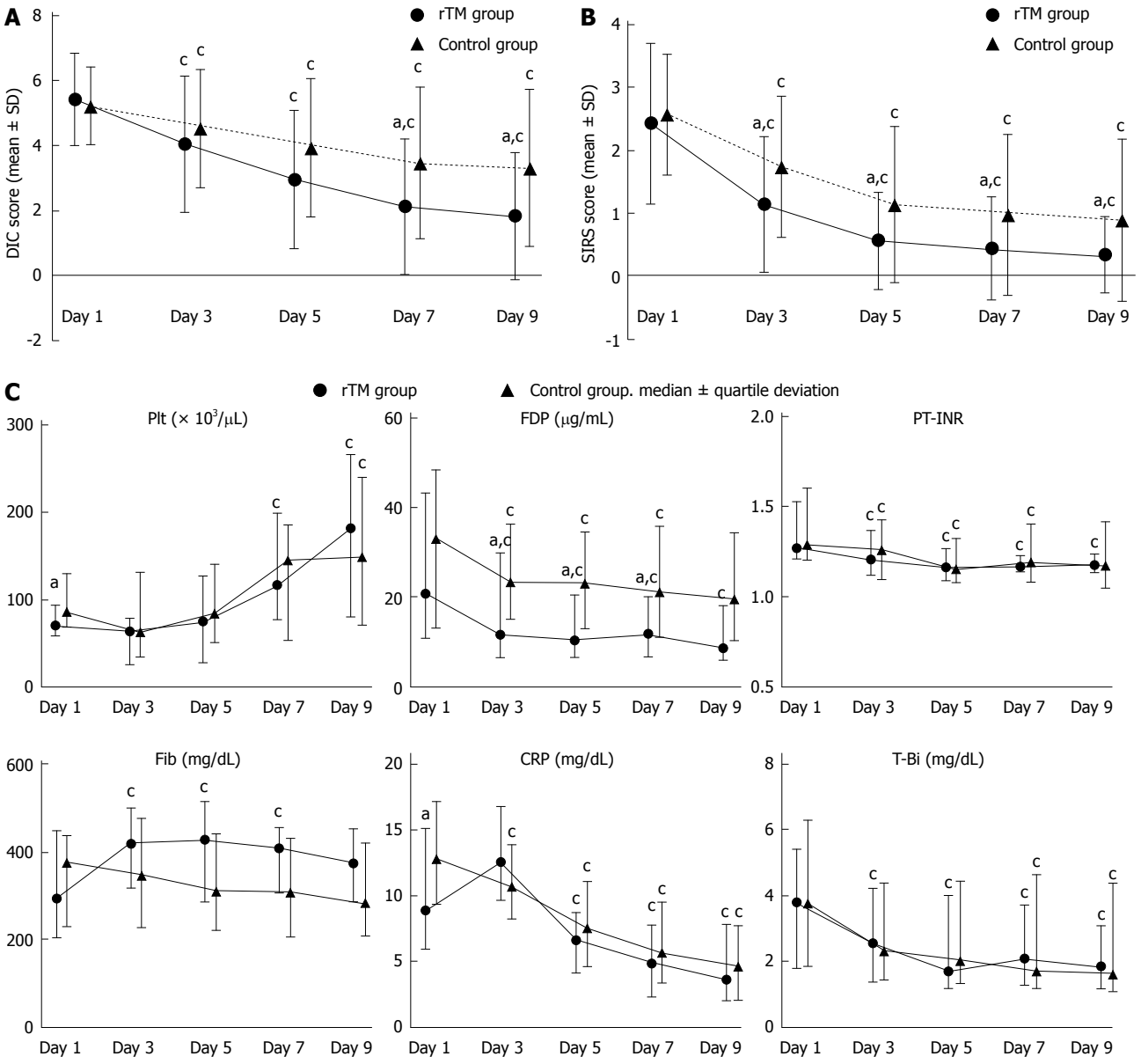

Figure 1 Comparison of the mean values of the disseminated intravascular coagulation scores (A), the systemic inflammatory response syndrome scores (B) and serum parameters between the recombinant human soluble thrombomodulin group and the control group (C). [a]*P* < 0.05 *vs* control group; [c]*P* < 0.05 *vs* baseline. DIC: Disseminated intravascular coagulation; rTM: Recombinant human soluble thrombomodulin; SIRS: Systemic inflammatory response syndrome; FDP: Fibrin/fibrinogen degradation products; PT-INR: Prothrombin time-international normalized ratio; Fib: Fibrinogen; CRP: C-reactive protein; T-Bil: Total bilirubin.

DIC[1-4]. In addition to this anticoagulant effect, rTM also elicits an indirect anti-inflammatory effect through activated protein C[9,11-13] and thrombin-activatable fibrinolysis[14,15]. Moreover, rTM exerting a direct anti-inflammatory effect by deactivating high mobility group box 1[16-18] and lipopolysaccharide[19] by binding to these molecules with the lectin-like domain of rTM. Thus, rTM has great potential as a drug for the treatment of infectious DIC.

However, the treatment of the underlying disease causing DIC is essential to achieve resolution of the pathological conditions that are associated with infectious DIC[5]. This is especially relevant in AC-induced DIC, where immediate biliary drainage can lead to prompt resolution of the DIC. Better therapies are needed, as there are still some DIC patients with poor outcomes; however, the usefulness of anti-DIC therapy with rTM remains unclear. Thus, we conducted the present study in patients with AC-induced DIC to evaluate the role of anti-DIC therapy with rTM by comparing outcomes between patients who did and did not receive rTM treatment.

Although there were no differences between the two groups in terms of age, sex, primary disease, severity of cholangitis, DIC score, or in the proportion of patients who underwent biliary drainage, the proportion of patients who received AT was significantly larger in the rTM group. However, the possibility of bias due to the therapeutic effects of AT must be taken into consideration when interpreting therapeutic outcomes in

Table 3 Factors associated with persistent disseminated intravascular coagulation (univariate analysis)

	Persistent DIC (n = 25)	Resolved DIC (n = 41)	P value	OR (95%CI)
Age (> 80 yr)	9	18	0.610	0.72 (0.26-2.0)
Female	7	16	0.431	0. 61 (0.21-1.8)
Primary disease (Malignant)	15	9	0.003	5.3 (1.8-16)
Severity of cholangitis (Severe)	23	37	1.000	1.2 (0.21-7.3)
DIC score (> 6)	12	15	0.442	1.6 (0.58-4.4)
SIRS score (> 3)	11	24	0.313	0.56 (0.20-1.5)
Without biliary drainage	14	3	< 0.001	16 (3.9-66)
Without rTM	19	17	0.010	4.5 (1.5-14)
Without AT	12	13	0.203	2.0 (0.71-5.5)
Without GM	5	17	0.107	0.35 (0.11-1.1)
Without NM	12	32	0.016	0.26 (0.088-0.76)
Without DS	22	37	1.000	0.79 (0.16-3.9)
Plt (< 80 × 10^3/μL)	12	24	0.452	0.65 (0.24-1.8)
FDP (> 25 μg/mL)	17	20	0.201	2.2 (0.79-6.3)
PT-INR	10	12	0.426	1.6 (0.57-4.6)
Fib (< 200 mg/dL)	7	5	0.186	2.8 (0.78-10)
CRP (> 15 mg/dL)	7	14	0.786	0.75 (0.25-2.2)
T-Bil (> 10 mg/dL)	4	3	0.412	0.49 (0.35-12)

DIC: Disseminated intravascular coagulation; SIRS: Systemic inflammatory response syndrome; rTM: Recombinant human soluble thrombomodulin; AT: Antithrombin; GM: Gabexate mesilate; NM: Nafamostat mesilate; DS: Danaparoid sodium; Plt: Platelet count; FDP: Fibrin/fibrinogen degradation products; PT-INR: Prothrombin time-international normalized ratio; CRP: C-reactive protein; Fib: Fibrinogen; T-Bil: Total bilirubin.

Table 4 Factors associated with persistent disseminated intravascular coagulation (multivariate analysis)

	P value	OR (95%CI)
Primary disease (Malignant)	0.055	3.9 (0.97-16)
Without biliary drainage	0.003	12 (2.3-60)
Without rTM	0.080	4.3 (0.84-22)
Without GM	0.680	1.5 (0.25-8.5)
Without NM	0.188	0.37 (0.083-1.6)
Fib (< 200 mg/dL)	0.403	2.2 (0.35-14)

rTM: Recombinant human soluble thrombomodulin; GM: Gabexate mesilate; NM: Nafamostat mesilate; Fib: Fibrinogen.

the rTM group. According to the Japanese guidelines for DIC treatment, which were prepared in 2009[5], AT is the most strongly recommended of all anti-DIC drugs. In the rTM group, which included patients who were treated in 2010 and thereafter, a higher frequency of AT use can be expected as a background condition. Because only a short time has elapsed since rTM became available, it is not included in the Japanese guidelines for DIC treatment. There have been many reports on the effectiveness of AT for the treatment of infectious DIC[20]. However, the KyberSept trial, reported in 2001[21], showed that the use of AT is not associated with decreased mortality, and the European guidelines for DIC treatment recommend restraint in the use of AT for the treatment of infectious DIC[22,23]. Our present univariate analysis identified only the use of rTM as a contributory factor in the successful treatment of DIC, while AT was not identified as such a factor. However, further studies are needed to determine the usefulness of AT for the treatment of AC-induced DIC; due to the retrospective nature of this study, we were unable to evaluate serum AT Ⅲ values in our patients.

The DIC resolution rate was significantly higher in the rTM group than in the control group, suggesting that rTM is highly effective for the treatment of AC-induced DIC. Although significant decreases in the DIC and SIRS scores from day 1 to day 3 were observed in both the rTM group and in the control group, a comparison between these two groups revealed that the DIC and SIRS scores had been significantly lower since days 7 and 3, respectively, in the rTM group and that greater improvements in the scores were observed in this group. The SIRS scores in particular were significantly improved in the early phase of treatment in the rTM group, which may be attributable to the anti-inflammatory effect of rTM[9,11-19]. With respect to the hematological findings, the control group showed significant improvements in Plt, CRP, and T-bil from day 1 to day 9, whereas the rTM group showed significant improvements in coagulation markers, such as FDP and PT-INR, in addition to Plt, CRP and T-bil. Although Plt levels on day 1 were significantly lower in the rTM group than in the control group, the Plt values on day 9 were higher in the rTM group. However, these differences did not reach statistical significance. Although there was no difference in FDP between the two groups on day 1, the levels of FDP were significantly lower from day 3 onward in the rTM group. These results suggest that rTM exerts a favorable anticoagulant effect. Thus, it is possible that in patients with AC-induced DIC, earlier and more marked resolution of the pathological condition may occur with the use of rTM.

There was no statistically significant difference in the mortality rate on day 28 between the two groups. However, the causes of death in all 4 patients in the rTM group were classified as malignant tumors, but the causes of death in 3 of the 10 deceased patients in

the control group were classified as being DIC-related. Based on these results, we can reasonably speculate that the resolution of DIC by rTM administration may have contributed to improved outcomes. In fact, there are reports on septic DIC describing reduced mortality at 28 d after the initiation of treatment with rTM[2,24,25]. In the present study, there were only 3 DIC-related deaths. To examine the effects of rTM on the improvement of the outcomes of patients with AC-induced DIC, multicenter studies with a larger sample size are needed.

In the present study, a multivariate analysis was performed to identify factors that contributed to persistent DIC. The absence of biliary drainage was identified as the only factor that contributed to persistent DIC. The treatment of the underlying disease causing DIC is considered to be the most important aspect of the treatment of infectious DIC[5], and the results of our study support this concept. Specifically, in patients with AC, a complete response is often achieved by biliary drainage[26,27], which is clearly the most important procedure for the clinical management of DIC. We advocate that biliary drainage be performed whenever possible. Furthermore, although the difference was not statistically significant, we observed that the non-use of rTM also tended to be associated with persistent DIC (P = 0.080, OR = 4.3, 95%CI: 0.84-22). It appears that treatment can be optimized by a combination of biliary drainage and the use of rTM. Moreover, our multivariate analysis revealed that the presence of malignant tumors also tended to be associated with persistent DIC, presumably because neoplastic as well as infectious DIC influenced the outcomes of patients in our study. Future studies are eagerly anticipated regarding the effects of rTM on neoplastic DIC due to solid cancers.

In conclusion, although biliary drainage for acute cholangitis is the most important treatment for AC-induced DIC, the use of rTM can lead to an earlier and more marked improvement in DIC and SIRS scores, which may improve clinical outcomes. However, to further examine the effects of rTM on the improvement of the outcomes of patients with AC-induced DIC, additional multicenter studies with a larger sample size are needed.

COMMENTS

Background

In acute cholangitis (AC)-induced disseminated intravascular coagulation (DIC), treatment for AC, including biliary drainage, can achieve resolution of the DIC. However, further improvements in treatment are needed, as there are still patients with poor outcomes.

Research frontiers

There have been several reports on the efficacy of recombinant human soluble thrombomodulin (rTM) for DIC that is associated with infection. However, in AC-induced DIC, the usefulness of anti-DIC therapy with rTM remains unclear.

Innovations and breakthroughs

The authors compared patients treated with rTM (rTM group) and without rTM (control group) to evaluate the role of anti-DIC therapy with rTM for AC-induced DIC. DIC resolution rates were higher in the rTM group (P < 0.01), and DIC scores were lower in the rTM group (P < 0.01). Multivariate analysis identified only the absence of biliary drainage as a contributor to the failure of DIC resolution (P < 0.01), and the non-use of rTM also tended to contribute to failure of DIC resolution (P = 0.08).

Applications

The add-on effects of rTM are anticipated in the treatment of AC-induced DIC, although biliary drainage for AC remains crucial.

Peer review

This paper is the first to demonstrate the effectiveness of rTM in cases of DIC due to acute cholangitis. Biliary drainage is the most effective procedure for the control of DIC, but rTM improves outcomes for patients. This retrospective study is original with solid data that is well analyzed.

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ORIGINAL ARTICLE

Retrospective Study

Mutations of pre-core and basal core promoter before and after hepatitis B e antigen seroconversion

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Abstract

AIM: To investigate the role of pre-core and basal core promoter (BCP) mutations before and after hepatitis B e antigen (HBeAg) seroconversion.

METHODS: The proportion of pre-core (G1896A) and basal core promoter (A1762T and G1764A) mutant viruses and serum levels of hepatitis B virus (HBV) DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen were analyzed in chronic hepatitis B patients before and after HBeAg seroconversion (n = 25), in those who were persistently HBeAg positive (n = 18), and in those who were persistently anti-HBe positive (n = 43). All patients were infected with HBV genotype C and were followed for a median of 9 years.

RESULTS: Although the pre-core mutant became predominant (24% to 65%, P = 0.022) in the HBeAg seroconversion group during follow-up, the proportion of the basal core promoter mutation did not change. Median HBV viral markers were significantly higher in patients without the mutations in an HBeAg positive status (HBV DNA: P = 0.003; HBsAg: P < 0.001; HB core-related antigen: P = 0.001). In contrast, HBV DNA (P = 0.012) and HBsAg (P = 0.041) levels were significantly higher in patients with the pre-core mutation in an anti-HBe positive status.

CONCLUSION: There is an opposite association of the pre-core mutation with viral load before and after HBeAg seroconversion in patients with HBV infection.

Key words: Seroconversion; Hepatitis B core-related antigen; Pre-core; Basal core promoter; Mutation; Hepatitis B surface antigen; Hepatitis B virus DNA

Core tip: The exact roles of pre-core (pre-C) and basal core promoter (BCP) mutations remain unclear before and after hepatitis B e antigen (HBeAg) seroconversion.

Here, although the pre-C mutant became predominant in the HBeAg seroconversion group during follow-up, the proportion of the BCP mutation did not change. Hepatitis B virus (HBV) viral markers were significantly higher in patients without the mutations in an HBeAg positive status. HBV DNA and hepatitis B surface antigen levels were higher in patients with the pre-C mutation in an anti-HBe positive status. Taken together, the association of the pre-C mutation on viral load appears to be opposite before and after HBeAg seroconversion in patients with HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma[1-4].

In the natural history of chronic HBV infection, seroconversion from hepatitis B e antigen (HBeAg) to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and the remission of hepatitis[5-7]. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion[8,9].

Several mutations in the HBV genome have been reported to associate with HBeAg seroconversion. When the pre-core (pre-C) and core genes in the HBV genome are transcribed and translated in tandem, HBeAg is produced and secreted into the circulation[10,11]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of detectable HBeAg[12,13]. The double mutations of A1762T and G1764A in the basal core promoter (BCP) of the HBV genome have also been shown to reduce HBeAg synthesis by suppressing the transcription of pre-C mRNA[14-16]. However, the detailed mechanisms of HBeAg seroconversion, including the involvement of mutations that decrease the production of HBeAg, have not been fully clarified. Orito *et al*[17] reported that a predominance of the pre-C mutation was correlated with anti-HBe, while BCP mutations were not associated with either anti-HBe or HBeAg. We previously uncovered that the pre-C and BCP mutations were frequently seen in patients with active replication after HBeAg seroconversion, but not in those with inactive replication[18], which suggested that HBeAg seroconversion was not associated with either mutation in such patients. Since the follow-up duration of these previous reports was limited, this study analyzed the changes in pre-C and BCP mutations among patients who were followed over a longer time course. Furthermore, we assessed the mutations not only in patients who seroconverted from HBeAg to anti-HBe, but also in those whose HBeAg or anti-HBe positive status did not change during follow-up.

MATERIALS AND METHODS

Patients

Three groups of patients with chronic hepatitis B who were categorized according to HBeAg/anti-HBe positive status were enrolled between 1985 and 2000. The subjects were selected retrospectively from a database of patients who had been followed for at least two years, had not received anti-viral therapy, such as nucleos(t)ide analogues, and whose stored serum samples were available from both the start and end of follow-up. We recruited only patients with HBV genotype C since this genotype is predominant in Japan and because the clinical significance of pre-C and BCP mutations differs among genotypes. The first group consisted of 18 patients whose HBeAg was persistently positive throughout the study period. The second group contained 25 patients in whom HBeAg seroconverted to anti-HBe. The third group was made up of 43 patients whose anti-HBe was persistently positive.

Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions a minimum of 6 mo apart in all patients before the start of follow-up. Tests for hepatitis C and human immunodeficiency virus antibodies were negative in all subjects. Patients who demonstrated accompanying hepatocellular carcinoma or signs of hepatic failure at the initial follow-up were excluded from the study.

Stored serum samples were kept frozen at -20 ℃ or below until assayed. This study was approved by the Ethics Committee of Shinshu University School of Medicine.

Conventional hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and anti-HBe, were tested using commercially available enzyme immunoassay kits (Fujirebio Inc., Tokyo, Japan)[19]. HBsAg was quantified[20] using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 8.9 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal and no signal detection was considered to be a negative signal. Six HBV genotypes (A-F) were

Table 1 Clinical and virological backgrounds among 3 groups of patients classified according to changes in hepatitis B e antigen/anti-hepatitis B e

Characteristic	HBeAg/anti-HBe status			*P* value
	Continuously +/- (*n* = 18)	From +/- to -/+ (*n* = 25)	Continuously -/+ (*n* = 43)	
Age (yr)[1]	44 (24-63)	37 (18-53)	51 (25-77)	< 0.001
Gender (M:F)	11:7	14:11	24:19	> 0.2
Follow-up period (yr)[1]	6.3 (2.1-14.6)	10.8 (2.0-23.7)	8.5 (2.2-16.6)	0.006
Genotype C[2]	18 (100)	25 (100)	43 (100)	1
Viral markers at first follow-up				
HBV DNA (log copies/mL)[1]	8.6 (5.7-> 8.9)	6.1 (< 2.1-> 8.9)	< 2.1 (< 2.1-8.2)	< 0.001
HBsAg (log IU/mL)[1]	4.6 (1.6-5.5)	3.6 (-0.9-4.6)	2.6 (< 0.05-4.3)	< 0.001
HBcrAg (log U/mL)[1]	> 6.8 (5.5->6.8)	6.8 (3.1-> 6.8)	3.0 (< 3.0-6.8)	< 0.001
Viral markers at final follow-up				
HBV DNA (log copies/mL)[1]	7.1 (< 2.1-> 8.9)	3.3 (neg.-6.2)	< 2.1 (neg.-7.0)	< 0.001
HBsAg (log IU/mL)[1]	3.3 (1.0-5.1)	2.8 (< 0.05-2.8)	1.3 (< 0.05-4.2)	< 0.001
HBcrAg (log U/mL)[1]	6.7 (4.4-> 6.8)	< 3.0 (< 3.0-6.2)	< 3.0 (< 3.0-5.3)	< 0.001

[1]Data are expressed as the median (range); [2]Data are expressed as a positive number (%). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis B core-related antigen.

evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al*[22]. Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc.) as described previously[23,24]. The HBcrAg assay simultaneously measured all antigens (e, core, and p22cr) encoded by the pre-C/core genes of HBV. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL with a quantitative range of 3.0 to 6.8 log U/mL.

Determination of pre-C and BCP mutations

The pre-C and BCP mutations were determined using nucleic acid samples extracted from 100 μL of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in wild type HBV and A1896 in the mutant were determined by mini-sequence reactions using labeled nucleotides that were complementary to either the wild type or mutant[25]. The results were expressed as percent mutation rates according to the definition by Aritomi *et al*[26] Samples were judged as positive for the pre-C mutation when the mutation rate exceeded 50% in the present study since the mutation rate was found to steadily increase to 100% once surpassing 50%[25].

The double mutation in the BCP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories)[25,26]. This kit detected T1762 and/or A1764 using the polymerase chain reaction (PCR) with primers specific for either wild type or mutant BCP. Results were recorded as wild, mixed, or mutant type. The pre-C and BCP mutations were tested at the start and end of follow-up with kits having manufacturer-established detection limits of 1000 copies/mL.

Full HBV genome sequencing

The nucleotide sequences of full-length HBV genomes were determined by a method reported previously[27]. Briefly, two overlapping fragments of an HBV genome were amplified by PCR, and then eight overlapping HBV DNA fragments were amplified by nested PCR. All necessary precautions to prevent cross-contamination were taken and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Version 3.1; Foster City, CA) with an automated ABI DNA sequencer (Model 3100, Applied Biosystems Carlsbad, CA).

Statistical analyses

The proportions of clinical factors were compared among groups using the χ^2 and Fisher's exact probability tests. Group medians were compared by means of the Mann-Whitney *U* test and Kruskal-Wallis test. The changes in proportions of the pre-C and BCP mutations between the study start and end points were compared using McNemar's test. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Patients

The clinical and virological backgrounds of the 3 groups are summarized in Table 1. Median age was lowest in patients with seroconversion, intermediate in those with persistent HBeAg, and highest in those with persistent anti-HBe. Gender ratio was similar among the 3 groups. Following our study design, all patients had HBV ge-

notype C.

Changes in pre-C and BCP mutations

The presence of the pre-C mutation could be evaluated in 60 (98%) of 61 HBeAg positive samples and 94 (85%) of 111 HBeAg negative samples. We were able to assess the existence of the BCP mutation in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples.

The changes in the proportion of the pre-C mutation between the start and end of follow-up are shown in Figure 1A. Wild type pre-C accounted for 94% of patients whose HBeAg was continuously positive at study onset and remained constant. Wild type pre-C was also predominant at the start of follow-up (76%, 19/25) in patients who experienced HBeAg seroconversion, but the mutant type had become predominant (P = 0.022) by the end of follow-up (65%, 15/23); 11 of 19 wild type pre-C patients converted to mutant type, while 2 of 6 patients with mutant type pre-C reverted to wild type. Mutant type pre-C accounted for 62% of the patients who were continuously positive for anti-HBe at study onset. Such patients with wild type pre-C at the start of follow-up tended to maintain this status (78%), although 22% of initially mutant type pre-C subjects had changed to wild type by the study end point (P = 0.687).

Of the 143 samples with determined BCP mutations, 34 (24%) were wild, 11 (8%) were mixed, and 98 (69%) were mutant types. Because few patients with mixed type BCP reverted to wild type in the present and past studies[18], samples were considered to be positive for the BCP mutation when they were either mixed or mutant type.

The changes in the proportion of the BCP mutation between the start and end of follow-up are shown in Figure 1B. Mutant type BCP accounted for 61% of patients whose HBeAg was continuously positive at study onset and remained constant. In patients who experienced HBeAg seroconversion, mutant type BCP was predominant at the start of follow-up (84%, 21/25) and remained so (80%, 16/20) until final follow-up; 3 of 4 patients with wild type BCP and 15 of 16 patients with mutant type BCP maintained their status throughout the study period. Mutant type BCP initially accounted for 82% of patients who were continuously positive for anti-HBe. Both wild (60%) and mutant (84%) types tended to remain constant until the study end point. When all points of measurement were counted for which both pre-C and BCP mutations were evaluated, the prevalence of the pre-C mutation (18%, 9/57) was significantly lower than that of the BCP mutation (82%, 42/57) in patients with persistent HBeAg (P < 0.001), as well as in subjects with persistent anti-HBe [62% (53/86) *vs* 78% (67/86), P = 0.030], albeit to a lesser degree.

Comparison of viral loads according to pre-C/BCP mutation and HBeAg/anti-HBe positive status

We next compared the serum levels of HBV DNA, HBsAg, and HBcrAg according to pre-C and BCP mutation and HBeAg and anti-HBe positive status (Figure 2). Both pre-C and BCP mutations could be evaluated in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples. HBV DNA levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status (P < 0.001) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status (P < 0.01). On the other hand, HBV DNA levels were significantly lower in patients without the pre-C mutation than in those with it in an anti-HBe positive status (P = 0.012).

A similar tendency to HBV DNA levels was observed for HBsAg levels. HBsAg levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status (P < 0.001) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status (P < 0.001). HBsAg levels were significantly higher in patients with the pre-C mutation than in those without it irrespectively of the existence of the BCP mutation (P = 0.041).

HBcrAg levels were significantly lower with presence of pre-C and/or BCP mutations in an HBeAg positive status (P < 0.05, respectively). HBcrAg levels were uniformly low regardless of the presence of mutations in anti-HBe positive status subjects.

Full genome sequences in patients with and without appearance of the pre-C mutation

Full HBV genome sequences were determined after HBeAg seroconversion in 6 patients who seroconverted without the appearance of the pre-C mutation. All patients were positive for BCP mutations: 1 subject had T1753G and C1766T mutations, although the other mutations reported by Okamoto *et al*[14] were not identified.

DISCUSSION

Although both pre-C and BCP mutations have been associated with HBeAg seroconversion by reducing the production of HBeAg[13-15], their manifestation patterns appear to be different[17]. In the present study, the BCP mutation was already prevalent during the HBeAg positive chronic hepatitis phase and approached 80% around the time of HBeAg seroconversion. On the other hand, the pre-C mutation clearly manifested following the time of seroconversion. These results indicate that the appearance of the pre-C mutation, but not the BCP mutation, is directly associated with seroconversion. It is noteworthy that a considerable number of patients experienced HBeAg seroconversion without evidence of the pre-C G1896A mutation. Furthermore, wild type pre-C remained unchanged in almost all patients whose anti-HBe was continuously positive. Thus, two types of HBeAg seroconversion may exist for chronic HBV in terms of the appearance or absence of the G1896A pre-C mutation. We previously speculated on the possible

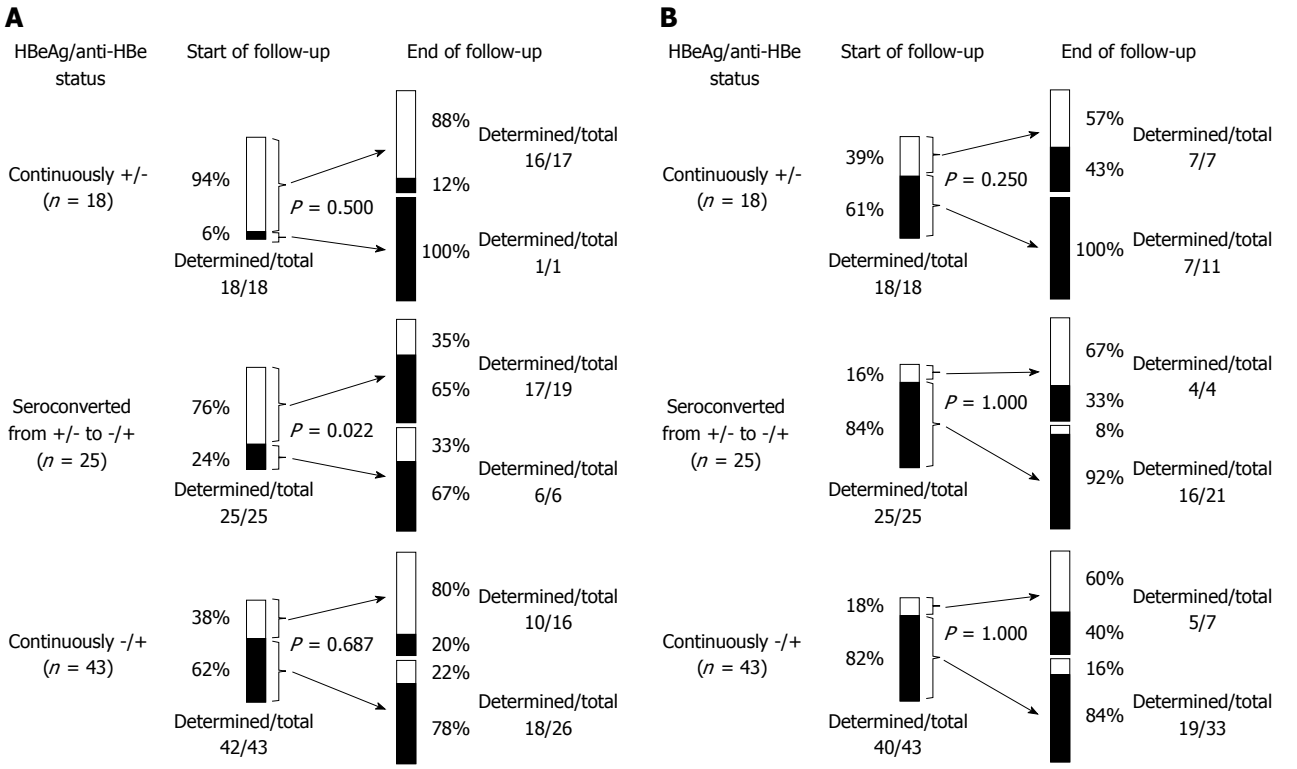

Figure 1 Comparison of changes in pre-core (A) and basal core promoter (B) mutation type among 3 groups of patients classified according to hepatitis B e antigen /anti-hepatitis B e positive status. A: A significant difference was seen in patients with hepatitis B e antigen (HBeAg) seroconversion (P = 0.022). One patient whose pre-core (pre-C) mutation was undetermined at the start of follow-up was wild type at the end point; B: Of the 3 patients whose basal core promoter (BCP) mutation was undetermined at the start of follow-up, 2 were wild type and 1 was undetermined at the end point. HBeAg: Hepatitis B e antigen.

existence of two seroconversion types in an analysis of HBV patients who experienced seroconversion[18]. Here, we were able to strengthen this notion by including patients who maintained an HBeAg or anti-HBe positive status in a study of longer duration. It should be noted that the absence of the pre-C G1896A mutation does not necessarily indicate the absence of mutations that halt HBeAg production; several patterns of mutations apart from G1896A have been associated with an HBeAg negative phenotype, such as point mutations in the ATG initiation region and deletion/insertion of nucleotides leading to premature termination[13]. Accordingly, we analyzed full genome sequences in 6 patients who seroconverted without the appearance of the pre-C mutation and uncovered T1753G and C1766T mutations in one subject[14] that might be associated with seroconversion. We observed that several patients reverted from mutant pre-C to wild type in the present report. As this important finding has not been confirmed by sequence analysis, we are planning to determine and compare entire genomic sequences using paired samples before and after HBeAg seroconversion in a future study.

We witnessed that serum HBV DNA was significantly lower in patients with the pre-C and/or BCP mutation in an HBeAg positive phase, which indicated that immune processes from the host to eliminate HBV were stronger in individuals with the mutations than in those without. This also supported the generally held belief that pre-C and BCP mutations appear as a result of host immune pressure[14]. Contrary to the HBeAg positive phase, HBV DNA was significantly higher in subjects with the pre-C mutation in an anti-HBe positive phase. Kawabe *et al*[28] have reported that patients with wild type pre-C demonstrate significantly lower viral loads and ALT levels than those with mutant pre-C among HBeAg negative patients with HBV genotype C infection. Collectively, these results imply that patients with the pre-C mutant have a higher potential to progress to hepa-titis after HBeAg seroconversion. This is consistent with the fact that HBeAg negative hepatitis is usually caused by HBeAg non-producing mutant strains of HBV. Indeed, viral replication seems to be considerably suppressed in patients with wild type HBV after achieving HBeAg seroconversion since this strain has the ability to produce HBeAg when actively replicated.

We adopted serum levels of HBsAg, HBcrAg, and HBV DNA in the present study as markers to estimate HBV replication activity. HBsAg and HBcrAg levels have been reported to reflect HBV cccDNA levels in hepatocytes[20,24,29]. HBsAg has also attracted attention as a useful predictor of treatment outcome by interferon and others[30]. Furthermore, the loss of HBsAg is an important indicator in the treatment of HBV carriers. HBcrAg assays simultaneously measure all antigens encoded by the pre-C/core genome, which include the HB core, e, and p22cr antigens, and have been reported to predict the clinical outcome of patients treated with nucleotide or nucleoside analogues[31]. HBsAg patterns according

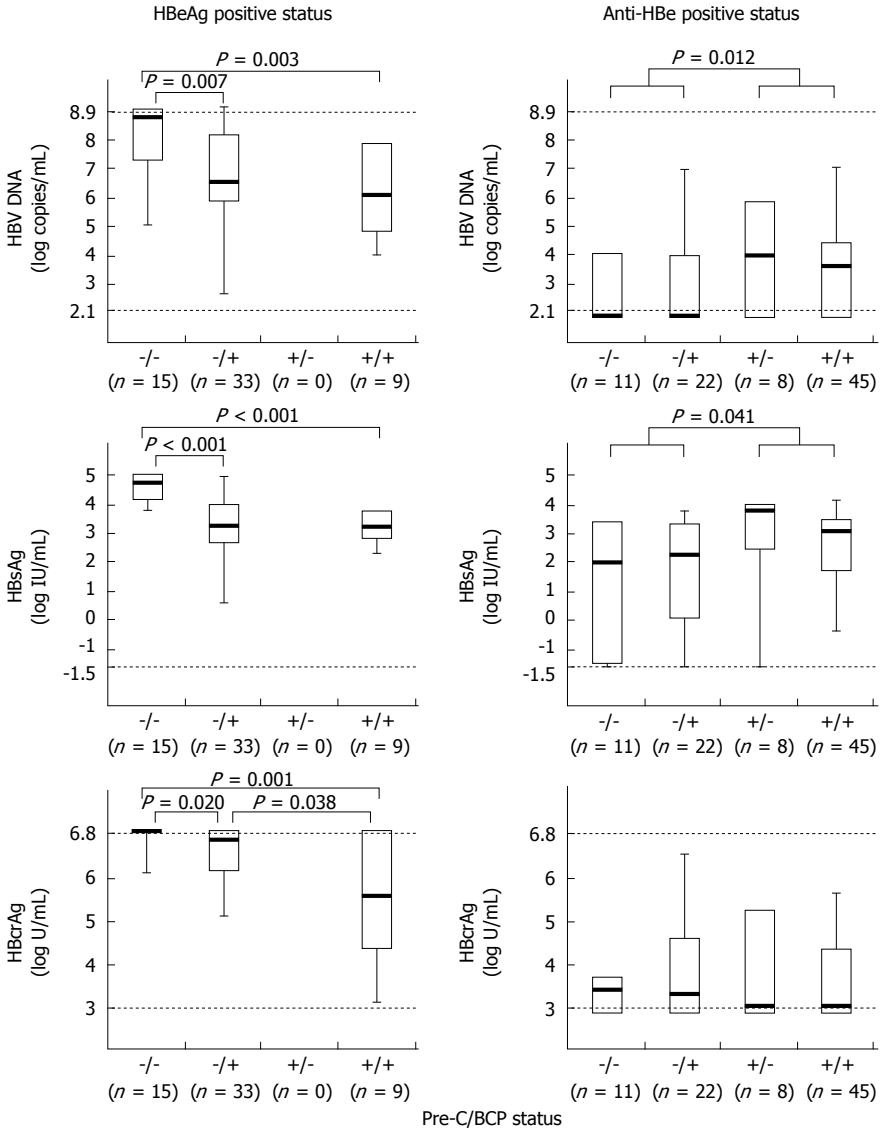

Figure 2 Comparison of serum hepatitis B virus DNA, hepatitis B surface antigen, and hepatitis core-related antigen levels among patients with wild (-/-) and mutant types of the pre-core and basal core promoter mutations. Fifty-seven of 61 samples obtained from HBeAg positive cases and 86 of 111 samples obtained from anti-HBeAg positive cases were eligible for analysis. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis core-related antigen; pre-C: Pre-core; BCP: Basal core promoter.

to HBeAg/anti-HBe and pre-C/BCP status were similar to HBV DNA patterns both in HBeAg and anti-HBe positive states; HBsAg was significantly lower in patients with pre-C and/or BCP mutations than in those with wild type pre-C but was significantly higher in patients with the pre-C mutation than in those without it in an anti-HBe positive state. These results confirmed that the pre-C mutation was oppositely associated with viral load in patients before and after HBeAg seroconversion. Since elevated levels of HBV DNA and HBsAg are related to a higher rate of hepatocarcinogenesis, pre-C mutation patterns appear to be clinically important, at least in the context of HBV genotype C patients. We witnessed that the patterns of HBcrAg were similar to those of HBV DNA in the HBeAg positive state but different in the anti-HBe positive state. This difference may reflect the fact that the main antigen measured by the HBcrAg assay is HBeAg.

In conclusion, our findings indicate that the association of the pre-C G1896A mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion. These observations may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

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COMMENTS

Background

Although pre-core (pre-C) and/or basal core promoter (BCP) mutations in the hepatitis B virus (HBV) genome have been reported to associate with hepatitis

B e antigen (HBeAg) seroconversion, the detailed mechanisms have not been fully clarified.

Research frontiers

In this study, the authors show that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion.

Innovations and breakthroughs

Recent reports have highlighted the importance of pre-C and BCP mutations of the HBV genome in association with HBeAg seroconversion. This study analyzed the changes in pre-C and BCP mutations in patients over a long follow-up period. The authors demonstrate that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection.

Applications

This study may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

Terminology

In the natural history of chronic HBV infection, seroconversion from HBeAg to anti-HBe is usually accompanied by a decrease in HBV replication and the remission of hepatitis. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion.

Peer review

The authors investigated the pre-C and/or BCP mutations before and after HBeAg seroconversion. They found that the association of the pre-C mutation on viral load is opposite in patients before and after HBeAg seroconversion. It is an interesting report. However there are several concerns.

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ORIGINAL ARTICLE

Retrospective Study

Histological mixed-type as an independent prognostic factor in stage I gastric carcinoma

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Abstract

AIM: To evaluate the clinicopathological features of mixed-type gastric cancer and their influence on prognosis of mixed-type stage I gastric cancer.

METHODS: We analyzed 446 patients who underwent curative gastrectomy for stage I gastric cancer between 1999 and 2009. The patients were divided into two groups: those with differentiated or undifferentiated cancer (non-mixed-type, n = 333) and those with a mixture of differentiated and undifferentiated cancers (mixed-type, n = 113).

RESULTS: The overall prevalence of mixed-type gastric cancer was 25.3% (113/446). Compared with patients with non-mixed-type gastric cancer, those with mixed-type gastric cancer tended to be older at onset (P = 0.1252) and have a higher incidence of lymph node metastasis (P = 0.1476). They also had significantly larger tumors (P < 0.0001), more aggressive lymphatic invasion (P = 0.0011), and deeper tumor invasion (P < 0.0001). In addition, they exhibited significantly worse overall survival rates than did patients with non-mixed-type gastric cancer (P = 0.0026). Furthermore, mixed-type gastric cancer was independently associated with a worse outcome in multivariate analysis [P = 0.0300, hazard ratio = 11.4 (1.265-102.7)].

CONCLUSION: Histological mixed-type of gastric cancer contributes to malignant outcomes and highlight its usefulness as a prognostic indicator in stage I gastric cancer.

Key words: Mixed-type gastric cancer; Histological type; Prognosis; Early gastric cancer

Core tip: Little is known about the clinical outcome of the histological mixed-type gastric cancer, which consists of differentiated and undifferentiated components. We evaluated the clinicopathological features of this cancer and their influences on the prognosis of

patients with mixed-type stage I gastric cancer.

Komatsu S, Ichikawa D, Miyamae M, Shimizu H, Konishi H, Shiozaki A, Fujiwara H, Okamoto K, Kishimoto M, Otsuji E. Histological mixed-type as an independent prognostic factor in stage I gastric carcinoma. *World J Gastroenterol* 2015; 21(2): 549-555 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/549.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.549

INTRODUCTION

Gastric cancer presents a variety of histological types, each of which shows different features. It is well known that the histological type is defined as one of the crucial factors for endoscopic treatment and lymphadenectomy in the treatment guidelines[1,2] and chemotherapy for advanced gastric cancer. In the early stage of gastric cancer, the histological type influences the extent of lymph node metastasis; the undifferentiated type, in particular, is one of the independent risk factors of lymph node metastasis[3-5]. In the advanced stage of gastric cancer, the histological type is an important factor that predicts prognosis, recurrence patterns, and chemosensitivity in patients[6-9]. Thus, the histological type of gastric cancer has been regarded as a crucial factor that may have a potentially useful role in determining treatment strategies.

However, gastric cancer tissues often present with histological heterogeneity; a cancer tissue does not always consist of a single histological type of tumor cell but sometimes consists of a mixture of several different types. Therefore, it is difficult for pathologists to diagnose accurately the histological differentiation state of the tissues of mixed-type gastric cancer due to the restricted tumor volume, even from several biopsy specimens. Moreover, there are some differences in the definitions of the histological type described by the 14th Japanese classification of gastric carcinoma (JCGC)[10] and the 7th tumour-node-metastasis (TNM) classification[11]. For example, mixed-type gastric cancer is classified based on the predominant component by the JCGC, whereas it is classified based on the weakest differentiated component by the TNM classification. Such differences in the definitions could give rise to disagreement about the recognition of the histological types, particularly of mixed-type gastric cancer.

In this study, therefore, we hypothesized that a mixture of differentiated and undifferentiated components itself might be associated with malignant clinical outcomes and poorer prognosis in patients undergoing curative gastrectomy for stage I gastric cancer. To verify this hypothesis, we evaluated prognosis relative to the extent of differentiated or undifferentiated components by comparing the clinicopathological features between two groups: the non-mixed-type, which consists of either differentiated or undifferentiated cancers; and the mixed-type, which is a mixture of differentiated and undifferentiated cancers. Our results suggest that the presence of mixed-type gastric cancer serves as an indicator of poor prognosis in patients with stage I disease and needs meticulous follow-up with clinical satisfaction.

MATERIALS AND METHODS

Patients and samples

Four hundred and forty-six patients with stage I gastric cancer diagnosed according to the criteria of the 14th JCGC[10] and the 7th TNM classification[11] were enrolled in this study. All patients underwent curative gastrectomy with radical lymphadenectomy in the Department of Digestive Surgery, Kyoto Prefectural University of Medicine, Japan between 1999 and 2009. Patients who underwent chemotherapy prior to surgery, had multiple lesions of gastric cancer, or both were excluded from this study. Median follow-up time was 63.0 mo. All patients were examined in the outpatient clinic by blood tests for carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9 every 3-6 mo after surgery, and annual computed tomography (CT) scans.

The resected stomach was opened and then placed on a flat board with the mucosal side up and was fixed in 10% buffered formalin solution. After fixation, tumors in the resected stomach were generally sectioned on the maximum cross-sectional plane parallel to the lesser curvature line based on the rules of the JCGC[10]. Tumors were sectioned in their entirety parallel to the reference line at intervals of 5 mm. The resected specimens were embedded in paraffin, and stained with hematoxylin and eosin. The clinicopathological features of these patients were obtained from hospital records based on the 14th JCGC[10] and the 7th TNM classification[11], excluding the definition of the histological type.

The histological types of resected tumor specimens were categorized into two major types: (1) expanding, intestinal or differentiated type; and (2) infiltrative, diffuse or undifferentiated type[12,13], based on the Japanese gastric cancer treatment guidelines 2010[1]. These state that the differentiated cancer includes papillary and tubular adenocarcinomas, which arise from the gastric mucosa with intestinal metaplasia, whereas the undifferentiated cancer includes poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma, which arise from ordinary gastric mucosa without intestinal metaplasia[14]. Quantitation of the relative extent of differentiated and undifferentiated components in resected specimens was determined by histological analysis by at least two pathologists in our hospital.

Subgroups based on histological differentiation state

No universal standard has existed regarding the definition of the histological type, particularly in mixed-type gastric cancer. To define histological type according to both the JCGC and the TNM classification, all of the gastric cancers were divided into four subgroups: (1) a group that consisted solely of a differentiated component (pure D group); (2)

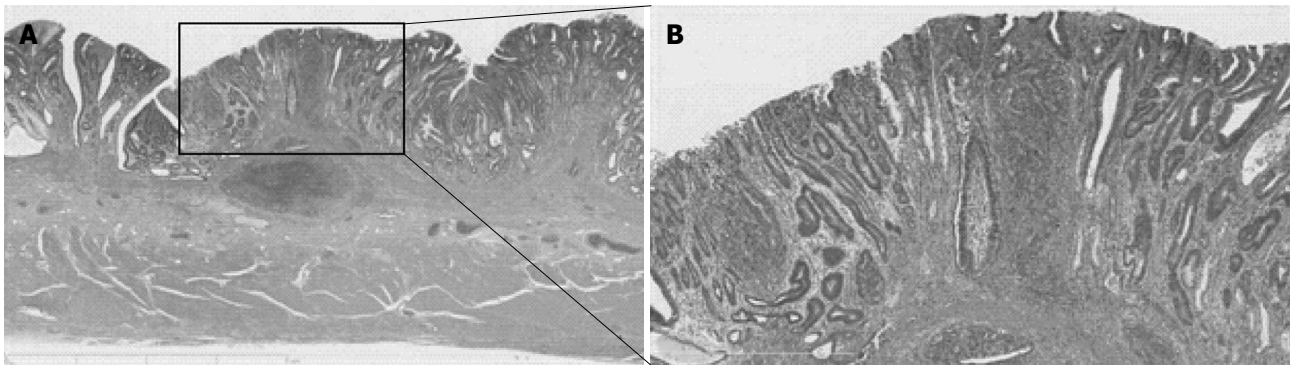

Figure 1 Representative case of mixed-type gastric carcinoma. A 68-year-old man, who underwent curative gastrectomy with lymphadenectomy for mixed-type gastric carcinoma (pT1bN0M0, 75 mm × 45 mm), died of peritoneal recurrence. A: Mixed-type gastric carcinoma consists predominantly of a differentiated component and has < 50% of an undifferentiated component; B: At a higher magnification.

a group that consisted predominantly of a differentiated component and has < 50% of an undifferentiated component (D > U group); (3) a group that consisted of > 50% of an undifferentiated component (U > D group); and (4) a group that consisted solely of an undifferentiated component (pure U group). According to the JCGC, the pure D and the D > U groups were classified as the differentiated type of gastric cancer, whereas the U > D and the pure U groups were classified as the undifferentiated type. On the other hand, according to the TNM classification, only the pure D group was classified as the differentiated type, and the remaining three groups as the undifferentiated type. Histological mixed-type gastric cancer consisted of both differentiated and undifferentiated components and belonged to the D > U or the U > D groups[15]. A representative case of mixed-type gastric cancer is shown in Figure 1.

Statistical analysis

The Fisher's exact probability test and χ^2 test were performed for categorical variables between two groups. Cause-specific death was recorded when death resulted from recurrent gastric cancer. The cumulative cause-specific overall survival rates were calculated by using the Kaplan-Meier method, and the log rank test was used to assess differences between clinical factors. Multivariate analysis using the Cox regression model was performed in order to identify significant contributors that were independently associated by univariate analysis. HRs are presented with 95%CIs. For all tests, $P < 0.05$ was considered to be statistically significant.

RESULTS

Distribution of gastric cancer patients among histological subgroups

The four subgroups of histological differentiation state consisted of the pure D group of 191 patients (43%), the pure U group of 142 patients (32%), the D > U group of 60 patients (13%), and the U > D group of 53 patients (12%). According to the criteria of the JCGC, 251 of the 446 patients (56%) were diagnosed with differentiated gastric cancer (the pure D and the D > U groups), whereas 195 patients (44%) were diagnosed with undifferentiated gastric cancer (the U > D and the pure U groups). In contrast, according to the criteria of the TNM classification, 191 of the 446 patients (43%) were diagnosed with differentiated gastric cancer (the pure D group), whereas 255 patients (57%) were diagnosed with undifferentiated gastric cancer (the D > U, U > D, and pure U groups). Moreover, the histological mixed-type was made up of the D > U and the U > D groups of 113 patients (25%), and the non-mixed-type consisted of the pure D and the pure U groups of 333 patients (75%).

Comparison of cause-specific survival rates of patients in each of the histological subgroups

Cause-specific survival curves showed that the 5-year survival rates of patients in the pure D, pure U, D > U, and U > D groups were 99.0%, 100.0%, 96.3%, and 96.1%, respectively (Figure 2). There was no significant difference in the cause-specific survival rates between the differentiated and undifferentiated gastric cancer groups as defined by the JCGC ($P = 0.7256$) and the TNM classification ($P = 0.3423$). The prognosis of patients with mixed-type gastric cancer was significantly worse than that of patients with non-mixed-type gastric cancer ($P = 0.0026$) (Figure 3). These data implied that a mixture of differentiated and undifferentiated components was associated with poor prognosis of patients with gastric cancer. Indeed, in differentiated gastric cancer as defined by the JCGC, the prognosis of patients in the D > U group was significantly worse than that of patients in the pure D group ($P = 0.0391$) (Figure 2). Similarly, in undifferentiated gastric cancer as defined by the JCGC, the prognosis of patients in the U > D group was significantly worse than that of patients in the pure U group ($P = 0.0231$) (Figure 2). These findings strongly suggest that a mixture of these two components in each histological type could be related to poor prognosis in

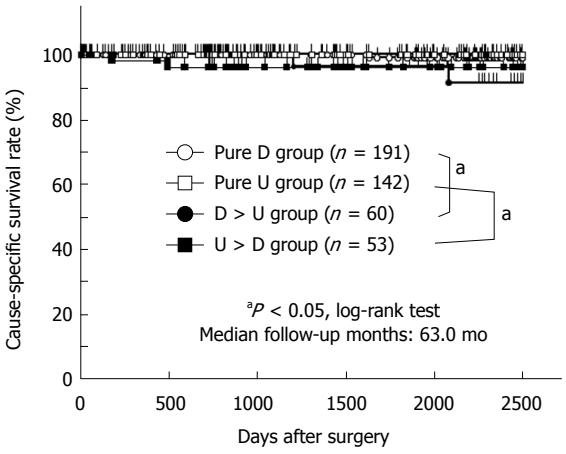

Figure 2 Comparison of cause-specific survival rates of patients in each of the histological subgroups. Four hundred and forty-six patients were divided into four histological subgroups, pure D, pure U, D > U, and U > D groups. Five-year cause-specific survival rates of the patients in each group were analyzed with the Kaplan-Meier method and log rank test. $P < 0.05$ was considered statistically significant. $^aP < 0.05$ *vs* control; D: Differentiated; U: Undifferentiated.

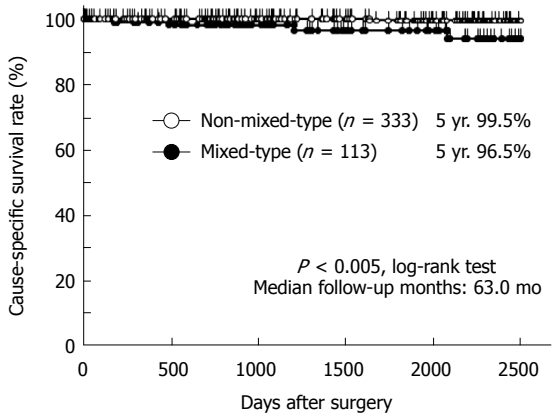

Figure 3 Comparison of cause-specific survival rates between the histological mixed-type and non-mixed-type in stage I gastric cancer. Five-year cause-specific survival rates in two groups, the mixed-type and the non-mixed-type, were analyzed with the Kaplan-Meier method and log rank test. $P < 0.05$ was considered statistically significant. $P < 0.05$ *vs* control.

patients with stage I gastric cancer.

Comparison of clinicopathological factors between the histological mixed-type and non-mixed-type in stage I gastric cancer

Clinicopathological factors were compared between the histological mixed-type and non-mixed-type of stage I gastric cancer (Table 1). Patients with the mixed-type cancer tended to be older at onset ($P = 0.1252$) and have a higher incidence of lymph node metastasis ($P = 0.1476$). They also had significantly larger tumors ($P < 0.0001$), more aggressive lymphatic invasion ($P = 0.0011$), and deeper tumor invasion ($P < 0.0001$).

Table 1 Association between clinicopathological characteristics and histological mixed-type in pathological stage I gastric cancer *n* (%)

	n	Histological type		*P* value
		Mixed-type	Non-mixed-type	
Sex	446	113	333	
Male	305	76 (67)	229 (69)	
Female	141	37 (33)	104 (31)	0.7651
Age (yr)				
< 65	233	52 (46)	181 (54)	
⩾ 65	213	61 (54)	152 (46)	0.1252
Location				
Upper	340	81 (72)	259 (78)	
Middle or Lower	106	32 (28)	74 (22)	0.1883
Histological type (JCGC)				
Differentiated	251	60 (53)	191 (57)	
Undifferentiated	195	53 (47)	142 (43)	0.4302
Macroscopic appearance (JCGC)				
Type 0	402	99 (88)	303 (91)	
Type 1-5	44	14 (12)	30 (9)	0.2977
Tumor size (mm)				
< 25	214	36 (32)	178 (53)	
⩾ 25	232	77 (68)	155 (47)	< 0.0001[1]
Venous invasion				
0	410	102 (90)	308 (92)	
1-3	36	11 (10)	25 (8)	0.4526
Lymphatic invasion				
0	370	82 (73)	288 (86)	
1-3	76	31 (27)	45 (14)	0.0011[1]
TNM classification				
pT categories				
T1a	11	34 (30)	185 (56)	
T1b	11	59 (52)	124 (37)	
T2	31	20 (18)	24 (7)	< 0.0001[1]
pN categories				
N0	427	105 (93)	322 (97)	
N1	19	8 (7)	11 (3)	0.1476

[1]Statistically significant values ($P < 0.05$). *P* values were calculated by χ^2 or Fisher's exact test. JCGC: Japanese classification of gastric carcinoma.

Investigation of the potential use of the histological mixed-type as a prognostic factor in patients with stage I gastric cancer

Cause-specific survival rates of 446 patients with stage I gastric cancer were evaluated by univariate and multivariate analyses (Table 2). By univariate analysis, the presence of venous invasion ($P < 0.0001$) or histological mixed-type cancer ($P = 0.0026$) was considered as a significant prognostic factor. Multivariate analysis using Cox regression procedures revealed that the presence of mixed-type cancer was an independent factor that could predict a poor prognosis [$P = 0.0300$, HR = 11.4 (1.265-102.7)].

DISCUSSION

Several studies have identified clinical features associated with the histological mixture of differentiated and undifferentiated components in gastric cancer[14-25]. However,

Table 2 Univariate and multivariate survival analyses using Cox's proportional hazard model in pathological stage I gastric cancer

Variables	Univariate[1]	Multivariate[2]			
	P value	HR	95%CI		*P* value
Sex					
Male *vs* female	0.5618				
Age (yr)					
≥ 65 *vs* < 65	0.1001				
Location					
U *vs* ML	0.8450				
Histological type (JCGC)					
Undiff *vs* Diff	0.7256				
Tumor size (mm)					
≥ 25 *vs* < 25	0.1918				
Venous invasion					
Positive *vs* Negative	< 0.0001[3]	13.513	2.252	83.33	0.0044[3]
Lymphatic invasion					
Positive *vs* Negative	0.1796				
pT-stage					
T2 *vs* T1	0.4781				
pN-stage					
N1 *vs* N0	0.6373				
Histological type					
Mixed *vs* Non-mixed	0.0026[3]	11.402	1.265	102.74	0.0300[3]

[1]Kaplan-Meier method, and statistical significance was determined by log-rank test; [2]multivariate survival analysis was performed using Cox's proportional hazard model; [3]Statistically significant values ($P < 0.05$). Diff: Differentiated; Undiff: Undifferentiated.

the prognostic effects of these components in patients with gastric cancer remain little known, particularly in the early stages. In the present study, we demonstrated that the presence of histological mixed-type gastric cancer was associated with old-age onset, large tumor, deep tumor invasion, lymphatic invasion, and lymph node metastasis in stage I gastric cancer. Furthermore, mixed-type cancer was observed to be an independent prognostic factor in stage I gastric cancer. These results clearly suggest that patients with mixed-type stage I gastric cancer should receive more careful attention.

With respect to clinical outcomes of mixed-type gastric cancer, only a few studies have reported that mixed-type cancer is associated with lymph node metastasis[16,17,20] and larger tumors[18]. Regarding the prognostic effects of the differentiation state, we previously demonstrated that mixed-type cancer was associated with poor prognosis in differentiated T1/T2 cancer, as defined by the JCGC[15]. On the other hand, we did not clarify the relevance of this factor in undifferentiated cancer, as defined by the JCGC. In this study, however, we elucidated that the prognosis of patients in the U > D group was significantly worse than that of patients in the pure U group ($P = 0.0231$). Including other results, our data suggest that a mixture of histologically differentiated and undifferentiated components itself contributes to malignant clinical outcomes and poor prognosis of patients with gastric cancer, whether or not undifferentiated components predominate.

Consistent with our results, Huh *et al*[23] demonstrated the significance of the histological mixed-type in the undifferentiated type of early gastric cancer. Specifically, the histological mixed-type of signet ring cell carcinoma was one of the independent risk factors of lymph node metastasis, and patients with the mixed-type of this carcinoma showed significantly lower survival rates than those of patients with the non-mixed-type of the same carcinoma[23]. That study also supports our finding that the clinical aggressiveness of histological mixed-type components in undifferentiated gastric cancer is independent of the predominance of the undifferentiated component. Furthermore, from the viewpoint of molecular pathology, mixed-type gastric cancer exhibited increased expression of Ki-67, extracellular matrix metalloproteinase inducer, and vascular endothelial growth factor proteins, which are involved in angiogenesis and cell proliferation[24], and enhanced the status of CpG island hypermethylation in tumor suppressive genes[25]. These data also support the idea that the histological mixed-type gastric cancer is clinically aggressive. However, further studies are needed to validate the detailed mechanisms by which histological mixed-type gastric cancer is more aggressive than non-mixed-type gastric cancer.

The histological type has been defined as one of the factors that determine limited treatments according to Japanese gastric cancer treatment guidelines[1]. Consequently, endoscopic submucosal dissection with narrow-band imaging magnifying endoscopy[26] and limited gastrectomy with laparoscopic surgery[27,28] have emerged as new, less-invasive technologies, and are widely accepted as limited treatments for early gastric cancer. However, it is true that there might be some problems in using the classification of histological types in clinical settings, because it is difficult for pathologists to diagnose accurately the histological differentiation, particularly in the histological mixed-type gastric cancer, let alone to diagnose the histological differentiation in biopsy specimens. Therefore, in order to apply histological differentiation to clinical settings, whether the histological mixed-type or not itself may also be a better factor to determine limited treatments, as proposed by recent studies and our results[15-18,20]. Indeed, it is not so difficult for pathologists to diagnose whether tumor specimens are histological mixed-type gastric cancer or not.

In conclusion, this is believed to be the first report to demonstrate that histological mixed-type cancer is related to malignant outcomes and poor prognosis in the early stage of gastric cancer, and to highlight its usefulness as an indicator of poor prognosis. Therefore, for patients with mixed-type gastric cancer, meticulous follow-up should be performed after curative gastrectomy, even at the early stage of this disease.

COMMENTS

Background

Several studies have identified clinical features associated with the histological mixture of differentiated and undifferentiated components in gastric cancer. However, little is known about the prognostic effects of these components in patients with gastric cancer, particularly in the early stages.

Research frontiers

This is believed to be the first report to demonstrate that histological mixed-

type cancer is related to malignant outcomes and poor prognosis in the early stage of gastric cancer, and to highlight its usefulness as an indicator of poor prognosis. Therefore, for patients with mixed-type gastric cancer, meticulous follow-up should be performed after curative gastrectomy, even at the early stage of this disease.

Innovations and breakthroughs

Four hundred and forty-six patients, who underwent curative gastrectomy for stage Ⅰ gastric cancer between 1999 and 2009, were enrolled in this study. The patients were divided into two groups: patients with either differentiated or undifferentiated cancer (non-mixed-type, n = 333) and patients with a mixture of differentiated and undifferentiated cancers (mixed-type, n = 113). The overall prevalence of mixed-type gastric cancer was 25.3% (113/446). Compared with patients with non-mixed-type gastric cancer, those with mixed-type gastric cancer tended to be older at onset (P = 0.1252) and have a higher incidence of lymph node metastasis (P = 0.1476). They also had significantly larger tumors (P < 0.0001), more aggressive lymphatic invasion (P = 0.0011), and deeper tumor invasion (P < 0.0001). In addition, they exhibited significantly worse overall survival rates than did patients with non-mixed-type gastric cancer (P = 0.0026). Furthermore, mixed-type gastric cancer was independently associated with a worse outcome in multivariate analysis [P = 0.0300, hazard ratio = 11.4 (1.265-102.7)].

Applications

These findings suggest that the histological mixed-type of gastric cancer contributes to malignant outcomes and highlight its usefulness as an indicator of poor prognosis in stage Ⅰ gastric cancer.

Terminology

Histological mixed-type gastric cancer: gastric cancer consists of both differentiated and undifferentiated components.

Peer review

This was a good descriptive study showing that the presence of mixed-type gastric cancer serves as an indicator of poor prognosis in patients with stage Ⅰ disease and requires meticulous follow-up with clinical satisfaction.

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ORIGINAL ARTICLE

Retrospective Study

Computed tomography and magnetic resonance imaging evaluation of lymph node metastasis in early colorectal cancer

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Abstract

AIM: To assess the role of computed tomography (CT) and magnetic resonance imaging (MRI) and establish imaging criteria of lymph node metastasis in early colorectal cancer.

METHODS: One hundred and sixty patients with early colorectal cancer were evaluated for tumor location, clinical history of polypectomy, depth of tumor invasion, and lymph node metastasis. Two radiologists assessed preoperative CT and/or MRI for the primary tumor site detectability, the presence or absence of regional lymph node, and the size of the largest lymph node. Demographic, imaging, and pathologic findings were compared between the two groups of patients based on pathologic lymph node metastasis and optimal size criterion was obtained.

RESULTS: The locations of tumor were ascending, transverse, descending, sigmoid colon, and rectum. One hundred and sixty early colorectal cancers were classified into 3 groups based on the pathological depth of tumor invasion; mucosa, submucosa, and depth unavailable. A total of 20 (12.5%) cancers with submucosal invasion showed lymph node metastasis. Lymph nodes were detected on CT or MRI in 53 patients. The detection rate and size of lymph nodes were significantly higher (P = 0.000, P = 0.044, respectively) in patients with pathologic nodal metastasis than in patients without nodal metastasis. Receiver operating curve analysis showed that a cut-off value of 4.1 mm is optimal with a sensitivity of 78.6% and specificity of 75%.

CONCLUSION: The short diameter size criterion of

⩾ 4.1 mm for metastatic lymph nodes was optimal for nodal staging in early colorectal cancer.

Key words: Early colon cancer; Lymph node metastasis; Computed tomography; Magnetic resonance imaging; Lymph node size

Core tip: This study is the first study on the imaging criterion of lymph node metastasis in early colorectal cancer. The results suggest that the detection rate and the size of lymph nodes (LNs) were significantly higher in patients with pathologic nodal metastasis. The optimal size criterion for LN metastasis was ⩾ 4.1 mm in early colorectal cancer.

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INTRODUCTION

Early colorectal carcinoma is defined as invasive carcinoma that has not spread in the direction continuity beyond the submucosal layer[1]. Recent advances in endoscopic instruments and techniques have increased the detection of small colorectal lesions, early colon cancers and adenomas[2]. The accurate assessment of lymph node (LN) metastasis in early colorectal cancer (CRC) is crucial for deciding appropriate treatment strategies such as endoscopic resection or surgery as well as for a prognostic factor[3]. Mucosal colorectal carcinoma is believed to have no potential for lymph node metastasis; however, reported incidences of lymph node metastasis in patients with submucosal carcinoma vary from 3.5% to 38%[4]. Risk factors for the lymph node metastasis of early colorectal cancers are deep invasion of depth, invasion of polyp stalk, poorly differentiated adenocarcinoma and presence of lymphovascular invasion[5-8]. However, these risk factors can be assessed only after the endoscopic removal of tumors and preoperative or pre-procedural diagnosis of lymph node metastasis with computed tomography (CT) or magnetic resonance imaging (MRI) is difficult. There have been a number of different imaging criteria for lymph node metastasis in colorectal carcinomas[9-22]; however, to the best of our knowledge, there have only been limited studies on early colorectal carcinoma. This study evaluates the imaging risk factors for LN metastasis in early CRCs and develops adequate diagnostic size criteria for LN metastasis in patients with early CRC.

MATERIALS AND METHODS

Participants

This study received study-specific institutional review board approval and a waiver of informed consent was obtained. Patients with surgically proven early colorectal cancer who underwent CT and/or MRI before radical resection (surgical excision of tumor mass and regional lymph node dissection) were retrospectively analyzed. We enrolled 160 patients (age range: 20-85 years; mean age: 59.7 years, male: 90, female: 70) for this study. Out of 160 patients, 141 patients underwent CT, 61 patients underwent MR examination and 42 patients underwent CT and MR examination before surgery. Lymph node size on MR was used for subjects who underwent both exams.

CT

All CT scans were obtained with one of the following commercially available multidetector CT scanners (Sensation 64; Siemens Medical Solutions, Erlangen, Germany), LightSpeed VCT; (GE Medical Systems, Milwaukee, Wisconsin). Each patient received 120 mL of nonionic contrast agent (iopromide, Ultravist 300; Bayer Schering Healthcare, Berlin, Germany) at a rate of 3 mL/s. Single-phase contrast-enhanced scans were obtained with a scanning delay of 75 s after Ⅳ administration of the contrast agent with 5 mm section thickness. The scanning parameters using Sensation 64 and LightSpeed VCT were: detector configuration, 0.6 × 32 mm/0.625 × 64 mm; nominal section thickness, 0.75/0.625 mm; beam pitch, 1/1; gantry rotation time, 0.5/0.5 s; reconstruction interval, 0.75/0.625 mm; tube voltage, 120/120 kV(p). Automated tube current modulation was routinely used for all patients and performed with a 64-detector row CT scanner (CareDose 4D, Siemens Medical Solutions with 210 image quality reference milliampere-s/AutomA, GE Healthcare with a noise index of 14). The data were reformatted in the axial and coronal planes with a 5-mm section thickness and a 5-mm interval. All the CT images were reviewed with a picture archiving and communication system workstation (Marotech 5.4, Seoul, South Korea).

MRI

MRI was performed using a 3T MR scanner (Magnetom Verio; Siemens Medical Solutions, Erlangen, Germany) with a phased-array multi-coil. Before MR scanning, approximately 50-100 mL of sonography transmission gel was administered for an appropriate distension of the rectum. The MR images were performed with the following sequences: A sagittal image was obtained with a T2-weighted fast spin-echo sequence. The perpendicular plane to the long axis of the rectal cancer was selected for axial scanning: oblique axial T1-weighted fast spin-echo sequence [TR/TE of 750/10; flip angle of 150; field of view (FOV) of 200 × 200 mm; matrix size of

320 × 224; 2 NEX; slice thickness of 5 mm with no gap; and acquisition time of 4 min 31 s] and oblique axial T2-weighted fast spin echo sequence (TR/TE of 4000/118; flip angle of 140; FOV of 200 × 200 mm; matrix size of 320 × 224; 2 NEX; slice thickness of 5 mm with no gap; acquisition time of 3 min 27 s). Diffusion-weighted MR images were acquired in the sagittal and oblique axial plane using a single shot-echo planar imaging technique with b of 0, 500 and 1000 s/mm^2; TR/TE of 6100/83; FOV of 200 mm; matrix size of 104 × 73; 2 NEX; slice thickness of 5 mm with no slice gap; and an acquisition time of 2 min 30 s. The contrast-enhanced T1-weighted image with fat suppression on the axial plane with TR/TE of 640/13; flip angle of 150; and slice thickness of 5 mm was obtained after an intravenous bolus injection of 0.1 mmol/kg Gadobutrol (Gadovist, Schering, Berlin, Germany) at a rate of 3 mL/s followed by a 25 mL saline flush.

Image interpretation

Two experienced board-certified radiologists (with 10- and 2-year experience in abdominal CT and MRI, respectively) were blinded for histological results and assessed preoperative CT and/or MR images for this study by consensus with access to the endoscopic findings of tumor location. Radiologists recorded the location and size of the mass, detectability of regional LNs 3 mm or larger and the size of regional LNs, when tumors were viewed on CT or MRI. Suspicious lymph nodes less than 3 mm were ignored because they cannot be differentiated from vascular structures or other non-specific soft tissue densities. The corresponding segment mentioned on endoscopy was evaluated for the evaluation of regional LNs, if the primary tumors were not visible; subsequently, radiologists evaluated the mesorectum for cases of rectal cancer and evaluated the sigmoid mesocolon for cases of sigmoid colon cancer. Two readers assessed primary tumor site detectability and the presence or absence of regional lymph nodes. They also measured the largest diameter of primary mass and short diameter of regional lymph nodes. A 3rd radiologist reviewed medical records for a clinical history of polypectomy (or endoscopic mucosal/submucosal resection) when patients were imaged and reviewed colonoscopic and histopathologic reports.

Pathology

A 3rd radiologist reviewed the pathologic reports for tumor depth of invasion, the presence or absence of lymph node metastasis and the number of metastatic lymph node. A 10-year experienced pathologist measured the size of 20 metastatic lymph nodes in short diameter.

Statistical analysis

Patients were divided into two groups based on pathologic lymph node metastasis. Differences in sex, age, tumor depth, tumor location, detectability of primary tumor site, detectability of regional lymph node, and lymph node size between those with lymph node metastasis and those without lymph node metastasis were tested. Univariate analysis was performed with Student's *t*-test for numerical data or the χ^2 test and Fisher's exact test for categorical data. Differences were considered significant when the *P*-value was less than 0.05. Receiver operating characteristic (ROC) analysis was used to obtain optimal lymph node size criterion. The area under the ROC curve was evaluated for diagnostic performance.

RESULTS

A total of 52 patients underwent CT or MRI after preoperative polypectomy and 17 endoscopic tumor resection sites were detected on CT or MRI among 52 patients. Out of 160 primary colonic masses, 77 tumors (mean size 2.7 cm; range: 0.8-8 cm) were detected on CT or MRI. The tumor location was divided into 5 groups; ascending (n = 17), transverse (n = 15), descending (n = 8), sigmoid colon (n = 50), and rectum (n = 70). A total of 160 early colorectal cancers were divided into 3 groups based on the pathological depth of tumor invasion; mucosa (n = 17), submucosa (n = 133) and depth unavailable (n = 10). A total of 20 (12.5%) cancers with submucosal invasion showed lymph node metastasis; however, there was no lymph node metastasis in any patients with mucosal cancer or with early cancer with unavailable depth. Recognizable lymph nodes were detected in 53 patients on CT or MRI near the primary tumor (Figures 1 and 2) or corresponding colonic segment of endoscopic finding (mean short diameter of lymph node; 4.5 mm, range: 3-14 mm). The average short diameter of 20 pathologic metastatic lymph nodes was 4.8 mm (range: 1.9-8.5 mm).

Only the detectability of regional lymph nodes and lymph node size showed a significant difference between nodal metastatic and non-metastatic groups (Table 1). The detection rate of lymph nodes was significantly higher (P = 0.000) in the pathologic nodal metastatic group (15/20) than in the non-metastatic group (38/140). The mean short axis diameter of the largest regional lymph nodes was significantly higher (P = 0.044) in the nodal metastatic group (5.686 mm) than in the non-metastatic group (4.121 mm).

ROC analysis was performed on the lymph node size parameter to obtain optimal diagnostic criterion to diagnose lymph node metastasis. The area under the ROC curve was 0.809 and the ROC curve showed that a criterion of 4.1 mm was optimal to diagnose lymph node metastasis, with a sensitivity of 78.6% and specificity of 75% (Figure 3).

DISCUSSION

The incidence of lymph node metastasis in early colon cancer is 7%-15% and the risk of lymph node metastases rises with advancing mural invasion into submucosa, up

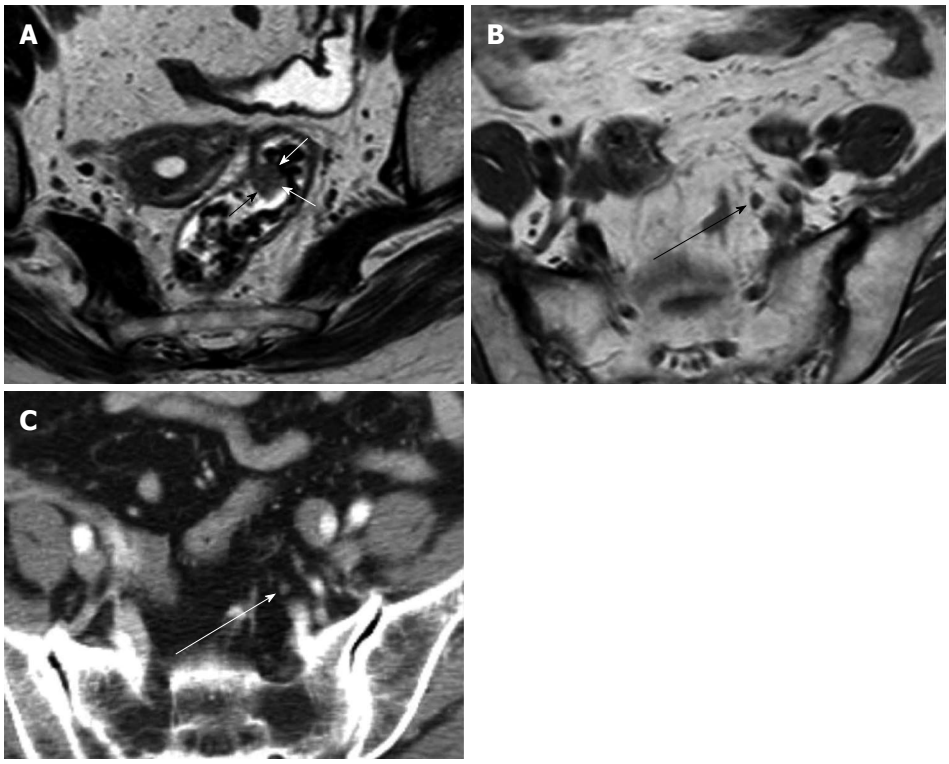

Figure 1 Early rectal carcinoma and lymph node metastasis on computed tomography and magnetic resonance imaging. A: Axial T2-weighted image shows polypoid rectal carcinoma (arrows); B: Axial T1-weighted image shows regional lymph node with 4.5 mm in short axis diameter (black arrow); C: Axial computed tomography scan shows the same regional lymph node (white arrow) as in B.

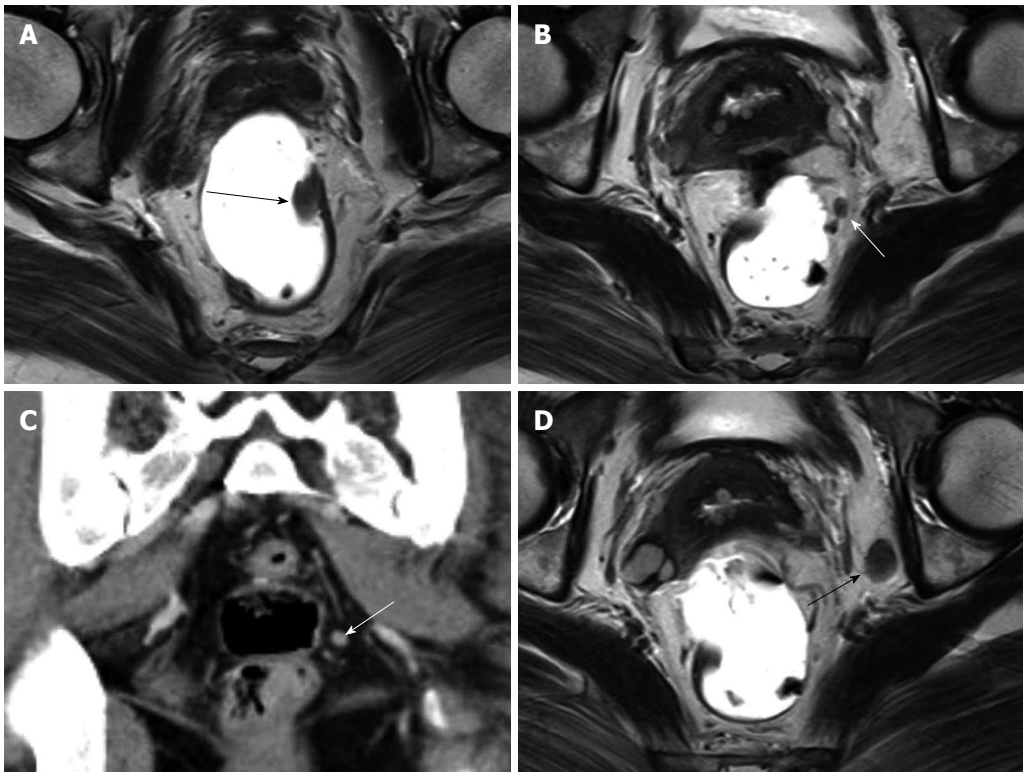

Figure 2 Early rectal carcinoma and lymph node metastasis on computed tomography and magnetic resonance imaging. A: Axial T2-weighted image shows polypoid rectal carcinoma (black arrow); B: Axial T2-weighted image shows perirectal lymph node (white arrow); C: Coronal computed tomography scan shows the same lymph node (white arrow); D: Axial T2-weighted image shows an enlarged left obturator lymph node (black arrow). These metastatic lymph nodes were one-to-one correlated pathologically.

Table 1 Demographic, imaging, and pathologic variables and lymph node metastasis

	Non-metastasis	Metastasis	*P* value
Sex (*n*)			0.547
M	80	10	
F	60	10	
Age (yr)	59.89 (20-85)	58.75 (38-81)	0.672
Tumor depth (*n*)			0.129
Mucosa	17	0	
Submucosa	113	20	
Tumor location (*n*)			0.756
Ascending	15	2	
Transverse	13	2	
Descending	6	2	
Sigmoid	45	5	
Rectum	61	9	
Detectability of primary tumor site (*n*)			
Yes	82	12	1.000
No	58	8	
Detectability of regional lymph node (*n*)			
Yes	38	15	0.000[1]
No	102	5	
Lymph node size (mm)	4.121 (3-6.5)	5.686 (4-14)	0.044[2]

[1]The detection rate of lymph nodes was significantly higher (P = 0.000) in the pathologic nodal metastatic group (15/20) than in the non-metastatic group (38/140); [2]The mean short axis diameter of the largest regional lymph nodes was significantly higher (P = 0.044) in the nodal metastatic group (5.686 mm) than in the non-metastatic group (4.121 mm).

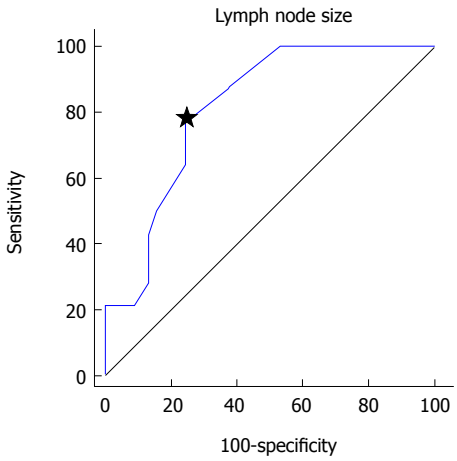

Figure 3 Receiver operating characteristic curve of short axis diameter. A criterion of 4.1 mm (star) showed optimal sensitivity (78.6%) and specificity (75%).

to 23%-38.5% in cases with the tumor depth of invasion over two thirds of submucosa[23]. Our study showed a 12.5% lymph node metastasis in early CRC patients, which is compatible with previous studies.

Conventional radiologic lymph node metastasis evaluation is traditionally based on nodal size and shape. There have been significant efforts to assess lymph node metastasis by size or other criteria. In previous reports, one of the general size criteria for nodal metastasis was 1cm in the short diameter[24]. Continuous technological advancements in CT and MR equipment made it possible to detect LNs as small as 5 mm on CT and MRI due to improved image resolution. Additionally, a higher rate of nodal micro metastases smaller than 5 mm has been reported in pathologic reports. Recent studies for rectal cancer presented 5 mm as an optimal size criterion, which showed moderate sensitivity (68%) and specificity (78%)[14-16,21,22]. However, a 5 mm cut-off value for LN metastasis in our early CRC group resulted in a sensitivity of 50% and a specificity of 81.6%. This too low sensitivity was caused by small-sized metastatic LNs. According to the pathologic reports, 30%-50% of metastatic lymph nodes in rectal cancer including both advanced and early cancer were smaller than 5 mm in size[21,22]. In the review of pathologic reports of our early CRCs, the average short diameter of metastatic lymph nodes was 4.8 mm and 56% of metastatic LN was less than 5 mm. Consequently, we need a modified size criterion for early colorectal cancer. The ROC curve analysis of short axis diameter of LNs in our study showed that a criterion of 4.1 mm or larger was optimal to diagnose LN metastasis in early CRC.

One obstacle to a smaller cut-off value of LN diameter was the ability of imaging modalities to detect small lymph nodes. Previous studies indicated that the smallest lymph nodes that can be detected were 5 mm for CT, and 3 mm for MR with using a spiral CT and a 1.5-T MRI[16,22]. Our study differentiated lymph nodes as small as 3 mm from other structures (such as blood vessels) due to the improved resolution of a 64 channel multi-detector row CT.

Unlike our study, a prior study showed no significant difference in the MRI detectability of LNs between nodal metastatic and non-metastatic groups[9]. The difference in findings also can be explained by the different incidence of enlarged reactive LNs between advanced and early cancers. The reactive LNs are usually not visible on imaging studies, but some enlarged reactive LNs can be

detected on CT/MRI. The prior study included mostly advanced stage rectal cancers (46/49) unlike our study which consisted of only early stage cancers. Therefore, we can presume that advanced cancers have a higher incidence of enlarged reactive LNs, which can be visualized on imaging, on the contrary to the lower incidence of enlarged reactive LNs in patients with early CRC. Consequently, we should pay more attention to lymph node detection for patients with early CRCs, because detectable LNs associated with early cancer are more likely metastatic rather than reactive LNs.

There are several limitations to our study. It was a retrospective study that may have various biases. Of particular note, most of metastatic LNs could not be correlated with the CT or MRI due to retrospective design. Nevertheless, our study showed significant difference in LN detectability of CT and MRI between metastatic and non-metastatic group. Therefore, our findings imply that LNs around early cancer on CT and MRI require attention in clinical practice. The number of metastatic lymph nodes were relatively small (n = 20); and may not reveal a real difference between the two groups. Selection bias is another inevitable component in a retrospective study. Although our study did not show significant difference among demographic data between nodal metastatic and non-metastatic groups, measurement error or inconsistent sensitivity of radiologists can be a confounding factor. To overcome this limitation, we need further prospective studies. In addition, we did not apply morphologic criteria, because morphologic evaluation was difficult in early CRC due to the small size of the lymph nodes. This study included patients without detectable primary tumors, and a limited evaluation of the corresponding mesocolon of primary tumor. However, our results indicated that the detectability of the primary tumor did not affect the pathologic LN metastasis. In clinical practice, radiologists should evaluate nodal status even in cases blinded to the exact location of the primary colon mass or when not visible due to small size, incomplete colon distention or post removal state. Our results indicated that a careful observation of the corresponding mesocolon segment is still important even in cases without detectable primary colon mass. The final limitation to our study was that the CT scanners used in our study were the most state-of-the-art equipment; 64 channel multi-detector CTs. Further studies are required for the reproducibility of small LN detection of 3 mm in lower-powered 8 or 16 channel CTs.

In conclusion, the advancement in imaging modalities will enable the detection of smaller lymph nodes and the establishment of more accurate size criteria for lymph node metastasis. Lymph node detectability and size of visible lymph nodes on CT/MR were significantly different between pathologic nodal metastatic and non-metastatic groups of patients with early colorectal carcinomas. A 4.1 mm short axis diameter criterion is believed optimal in the CT/MR evaluation of regional lymph node metastasis in patients with early colorectal carcinoma.

COMMENTS

Background

The accurate assessment of lymph node (LN) metastasis in early colorectal cancer (CRC) is crucial for deciding appropriate treatment strategies such as endoscopic resection or surgery as well as for use as a prognostic factor. Recent imaging studies considered 5 mm as an optimal size criterion for LN metastasis in colorectal cancer. However, previous studies have not focused on early colorectal carcinoma.

Research frontiers

Continuous development of computed tomography (CT) and magnetic resonance imaging (MRI) made it possible to detect small LNs by CT and MRI due to improved image resolution. This study evaluates the radiologic risk factors for LN metastasis in early CRCs and develops adequate diagnostic size criteria for LN metastasis in patients with early CRC.

Innovations and breakthroughs

Most of previous studies for evaluation of radiologic criteria included both advanced and early colorectal cancer, but the portion of included early cancer was very small. The size criteria (approximately 0.5-1 cm) for diagnosing metastatic LNs in previous study showed low to intermediate sensitivity, because the ranges of sizes of metastatic and non-metastatic LNs were overlapped. However, the sizes of LNs in the metastatic group and the non-metastatic group were as significantly different in this study. Authors presumed that this different result from previous study was because their study included only early CRCs. They concluded that the differentiated criteria for early CRC is needed and evaluated optimal size criteria for LN metastasis. The newly suggested size criterion for metastatic LNs in early CRC is 4.1 mm, slightly smaller compared to the existing criterion for both advance and early CRC.

Applications

For the CT or MRI evaluation of early colorectal cancer, an application of a differentiated size criterion from advanced cancer for LN metastasis could be helpful in management planning for early CRC.

Peer review

This is a good retrospective study in which the authors assessed the role of CT and MRI to establish the imaging criteria of LN metastasis in early colorectal cancer. The results are interesting and suggest that the detection rate and the size of LNs were significantly higher in patients with pathologic nodal metastasis. The optimal size criterion for LN metastasis was ⩾ 4.1 mm in early colorectal cancer.

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ORIGINAL ARTICLE

Retrospective Study

Impression of prognosis regarding pathologic stage after preoperative chemoradiotherapy in rectal cancer

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Abstract

AIM: To ascertain pathologic stage as a prognostic indicator for rectal cancer patients receiving preoperative chemoradiotherapy (PCRT).

METHODS: Patients with mid- and low rectal carcinoma (magnetic resonance imaging - based clinical stage Ⅱ or Ⅲ) between 2000 and 2009 and treated with curative radical resection were identified. Patients were divided into two groups: PCRT and No-PCRT. Recurrence-free survival (RFS) was examined according to pathologic stage and addition of adjuvant treatment.

RESULTS: Overall, 894 patients were identified. Of these, 500 patients received PCRT. Adjuvant chemotherapy was delivered to 81.5% of the No-PCRT and 94.8% of the PCRT patients. Adjuvant radiotherapy was given to 29.4% of the patients in the No PCRT group. The 5-year RFS for the No-PCRT group was 92.6% for Stage Ⅰ, 83.3% for Stage Ⅱ, and 72.9% for Stage Ⅲ. The 5-year RFS for the PCRT group was 95.2% for yp Stage 0, 91.7% for yp Stage Ⅰ, 73.9% for yp Stage Ⅱ, and 50.7% for yp Stage Ⅲ.

CONCLUSION: Pathologic stage can predict prognosis in PCRT patients. Five-year RFS is significantly lower among PCRT patients than No-PCRT patients in pathologic stage Ⅱ and Ⅲ. These results should be taken into account when considering adjuvant treatment for patients treated with PCRT.

Key words: Preoperative; Chemoradiotherapy; Rectal cancer; Pathologic stage; Prognosis

Core tip: Strictly speaking, there is no common objective guideline to predict prognosis and give adjuvant treatment according to risk stratification. Patients who show good response were thought to have good prognosis. However, expected value of recurrence-free survival or recurrence rate was not suggested

especially in patients who did not show good response to patients receiving preoperative chemoradiotherapy (PCRT). In addition, how to measure the response level was variable. The present study suggests impression of prognosis based on pathologic stage, which is objective, after PCRT and radical resection and show stage-by-stage comparison with those without PCRT to give impression of prognosis by using familiar stage-based prognosis.

Hwang K, Park IJ, Yu CS, Lim SB, Lee JL, Yoon YS, Kim CW, Kim JC. Impression of prognosis regarding pathologic stage after preoperative chemoradiotherapy in rectal cancer. *World J Gastroenterol* 2015; 21(2): 563-570 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/563.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.563

INTRODUCTION

Pathologic staging is used to select high-risk patients for adjuvant treatment to reduce disease recurrence and improve survival[1]. Preoperative chemoradiotherapy (PCRT) followed by radical resection is the standard treatment for patients with clinical stage Ⅱ-Ⅲ rectal cancer. A tumor down-staging rate of 40%-80% and a pathologic complete response (pCR) rate of 10%-25% can be achieved after PCRT[2-6]. Patients achieving tumor down-staging after preoperative therapy tend to have better local control and increased survival. Conversely, patients with persistent nodal disease after chemoradiation have a very poor prognosis[7-9]. However, there is uncertainty concerning the difference in prognosis according to pathologic stage for patients treated with PCRT. In addition, although several studies have shown that pathologic stage after PCRT (yp stage) followed by radical resection is a significant prognostic indicator, prognostic information is not usually used to inform post-surgical clinical practice for PCRT patients in contrast to patients with rectal cancer who are not treated with PCRT[10-12].

The National Comprehensive Cancer Network guidelines recommend that all patients undergoing PCRT receive postoperative chemotherapy regardless of the pathologic results[13]. This recommendation is based on preoperative clinical staging. Although adjuvant chemotherapy is regarded as the standard treatment[11,14-17] irrespective of the final pathologic stage[11,14-17], evidence supporting the routine use of adjuvant chemotherapy (according to pretreatment clinical stage) for patients with advanced rectal cancer after PCRT is lacking.

In addition, though it has been suggested that patients who do not respond to PCRT have a poor prognosis, the extent of this effect is not clear.

Some studies have indicated that the final pathologic stage is more predictive of long-term outcome (*e.g.*, disease-free survival) than the preoperative clinical stage or degree of down-staging[7,11,18-20]. Thus, it appears plausible to use pathologic stage as a criterion for adjuvant chemotherapy and for formulating a prognosis.

In the present study we compared the prognosis (based on pathologic stage) of patients with advanced rectal cancer who received PCRT with that of patients not treated with PCRT, and evaluated the usefulness of yp stage as an outcome predictor and guideline for adjuvant treatment.

MATERIALS AND METHODS

Patient identification

Patients with biopsy-proven mid- and low rectal cancer who were treated with curative surgery at Asan Medical Center between 2000 and 2009 were identified from the institutional colorectal cancer patient database and tumor registry. Cases in which the lower border of the tumor was located ⩽ 5 cm from the anal verge (as assessed by proctoscopy or digital rectal examination) were defined as low rectum, and those located > 5 cm, ⩽ 10 cm from the anal verge were defined as mid-rectum. Patients with concurrent distant metastasis, concurrent inflammatory bowel disease, hereditary colorectal cancer syndromes, or concurrent malignancy, or those requiring urgent surgery, or with a prior history of immunotherapy or radiotherapy to the pelvis or a prior history of malignancy other than non-skin melanoma or *in situ* cervical cancer, were excluded. Patients with no identifiable exact clinical stage and pathologic stage were also excluded. The study was approved by the Asan Institutional Review Board.

Clinical staging, pathologic evaluation, and treatment

Preoperative clinical staging was based on magnetic resonance imaging (MRI). MRI diagnosis of T3 lesions was based on the presence of an tumor signal intensity extending through the muscle layers into the perirectal fat, with a broad-based bulging configuration, and continuous with the intramural portion of the tumor. A clinical T4 lesion was defined as direct invasion to an adjacent organ. Positive lymph node (LN) status was ascertained from signal intensity, border characteristics, irregular contour, or heterogeneous texture. In addition, diameter larger than 5 mm was used as a predictor of LN positivity. Upfront resection was recommended for patients with obstructive lesion. For patients with cT3-4 and/or N+, tumor involvement to mesorectal fascia was checked using MRI or CT. When it is possible to get clear mesorectal margin by upfront surgery, the current disease status, possible advantage of PCRT, and expected response rate was explained to patient, and patient involve in selection of treatment plan. If mesorectal fascia involvement was suspected, PCRT was recommended primarily. The PCRT regimen comprised pelvic external beam radiation (45 Gy given in 25 fractions over 5 wk) followed, in most cases, by a boost of 5.4 Gy (in 5 fractions) applied directly to the tumor. This boost was delivered as a second daily fraction during the final week of treatment, taking the

Table 1 Characteristics of patients receiving and not receiving preoperative chemoradiotherapy *n* (%)

	No-PCRT (*n* = 394)	PCRT (*n* = 500)	*P* value
Age (yr), median (range)	60 (52-68)	57 (49-64)	< 0.001
< 50	72 (18.3)	124 (24.8)	
50-65	183 (46.4)	269 (53.8)	
> 65	139 (35.3)	107 (21.4)	
Gender			0.028
Male	236 (59.9)	335 (67.0)	
Female	158 (40.1)	165 (33.0)	
Location			< 0.001
Mid-rectum	277 (70.3)	175 (35.0)	
Low rectum	117 (29.7)	325 (65.0)	
Sphincter preservation	358 (90.9)	372 (74.4)	< 0.001
Among patients with low rectum	83 (70.9)	203 (62.5)	0.053
Clinical stage			0.86
Ⅱ	21 (5.3)	28 (5.6)	
Ⅲ	373 (94.7)	472 (94.4)	
Pathologic stage[1]			
0	-	83 (16.6)	
Ⅰ	97 (24.6)	128 (25.6)	
Ⅱ	145 (36.8)	135 (27.0)	
Ⅲ	152 (38.6)	154 (30.8)	
Number of harvested lymph nodes	16 (11-22)	13 (9-17)	< 0.001
Length of distal resection margin (cm)	2.2 (1.4-3.3)	2.4 (1.4-3.8)	0.64
Adjuvant chemotherapy[1]	321 (81.5)	474 (94.8)	< 0.001
Adjuvant radiotherapy	116 (29.4)		
Follow-up duration (mo)	60 (39-80)	56 (43-68)	0.54

[1]The pathologic stage for the PCRT group was based on yp stage. PCRT: Preoperative chemoradiotherapy.

cumulative radiation dose to 50.4 Gy. Most of the patients were treated with concurrent chemotherapy comprising 5-fluorouracil and leucovorin (FL) and capecitabine and were included in the PCRT group. FL was delivered *via* two intravenous bolus injections of 5-fluorouracil (375 mg/m^2 per day) and leucovorin (20 mg/m^2 per day) for 3 d during the first and fifth weeks of radiotherapy. Capecitabine (825 mg/m^2) was given twice daily (orally) during radiotherapy. Surgery was performed 6-8 wk after the completion of radiotherapy according to principle of total mesorectal excision.

Adjuvant chemotherapy is recommended for all No-PCRT patients with pathologic stage Ⅲ disease and those with stage Ⅱ with risk factors such as lymphovascular invasion, perineural invasion, preoperative obstruction, and perforation. Adjuvant chemotherapy followed by radical resection is recommended for all medically-fit PCRT patients. The usual adjuvant treatment comprised FL for 4 cycles monthly or capecitabine for 6 cycles. Oxaliplatin regimens were delivered at the discretion of the attending physician. In some cases protocol-based concurrent chemotherapy included the addition of irinotecan or bevacizumab. Postoperative follow-up comprised routine physical examination and carcinoembryonic antigen (CEA) assays every 3-6 mo, and cross-sectional imaging every 6-12 mo over a period of 5 years. Colonoscopy was performed at 6-12 mo after surgery and then every 2-3 years thereafter.

Recurrence-free survival (RFS) was used as the cancer recurrence end point. RFS was defined as the time from surgery to any type of tumor recurrence. Patients who died without evidence of confirmed tumor recurrence were censored at the time of death.

Statistical analysis

Non-parametric data were compared using the Wilcoxon rank sum test. Categorical data were summarized according to frequency within each cohort and compared using the χ^2 test. Kaplan-Meier survival analysis was used to determine 5 year RFS, and the log rank test was used to compare RFS with pathologic tumor stage. Cox proportional hazards regression analysis was employed to examine the relationship between various factors and treatment effects. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed with SPSS (Version 21.0; IBM statistics, New York, NY).

RESULTS

Patient characteristics

A total of 894 patients who underwent curative resection for cT3-4 or N+ (MRI based) mid- and low-rectal cancer during the study period were eligible. Of these, 500 (55.9%) received PCRT. The median patient age was 59 [interquartile range (IQR): 50-66] years, and the majority (63.9%) was male. The median distance of the tumor from the anal verge was 5 (IQR: 3-8) cm, and 49.4% of the patients had low rectal cancer. The median radiation dose was 50.4 (IQR: 45-52.5) Gy. Sphincter-preserving resection was performed for 730 (81.7%) of the patients. The patients in the PCRT group were younger than those in the No-PCRT group, there were more males, and most had a low rectum tumor (Table 1). Sphincter-sparing surgery was performed more frequently in the No-PCRT group. Taking into account only those patients with a low rectal tumor, the sphincter-sparing surgery rates were 62.5% for the PCRT group and 70.9% for the No-PCRT group ($P = 0.05$; Table 1). Fewer lymph nodes were excised from patients in the PCRT group than from those in the No-PCRT group (median, 13 *vs* 16, $P < 0.001$).

Adjuvant chemotherapy was administered to 81.5% of the patients in the No-PCRT group and to 94.8% of those in the PCRT group. The adjuvant chemotherapy regimen administered to the PCRT group comprised FL (25.7%) or capecitabine (63.4%).

Recurrence and survival

Overall, 5-year RFS was higher in the No-PCRT (80.8%) than the PCRT (74.9%) group ($P = 0.01$). According to clinical stage, 5-year RFS did not differ between the No-PCRT and PCRT group. In clinical stage Ⅱ, 5-year RFS was 79.4% with PCRT and 81% with No-PCRT ($P = 0.66$). In clinical stage Ⅲ we evaluated 5-year RFS

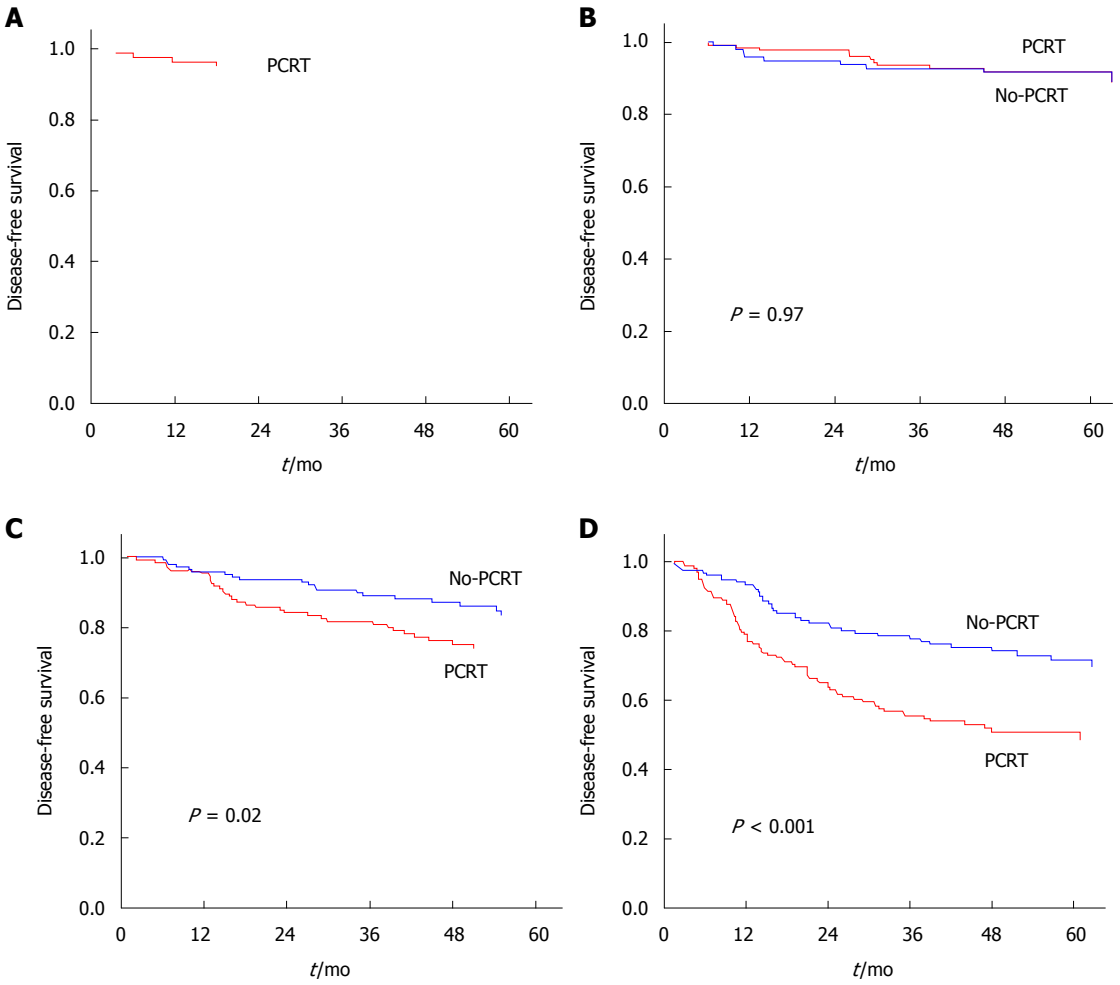

Figure 1 Recurrence-free survival according to pathologic stage. A: p Stage 0; B: p Stage Ⅰ *vs* yp Stage Ⅰ; C: p Stage Ⅱ *vs* yp Stage Ⅱ; D: p Stage Ⅲ *vs* yp Stage Ⅲ. PCRT: Preoperative chemoradiotherapy.

according to cT category. For cT3N+, 5-year RFS was 75.3% with PCRT and 80.7% with No-PCRT ($P = 0.1$). For cT4N+, it was 61% with PCRT and 63.4% with No-PCRT ($P = 0.51$).

5-year RFS rates (stratified according to yp stage and p stage) were: 95.2% for yp stage 0; 91.7% for yp stage I; 92.6% for p stage Ⅰ; 73.8% for yp stage Ⅱ; 83.3% for p stage Ⅱ; 50.7% for yp stage Ⅲ; and 72.9% for p Stage Ⅲ (Figure 1).

Recurrence-free survival and adjuvant chemotherapy for patients treated with PCRT

Forty patients in the PCRT group received second-line adjuvant chemotherapy: 37 received oxaliplatin-based chemotherapy, 2 received irinotecan-based chemotherapy, and one had target agent. Twenty-three patients with yp Stage Ⅲ (14.9%) among the patients who received 1st line chemotherapy-based PCRT received a second-line adjuvant chemotherapeutic regimen that was different from the preoperative concurrent chemotherapeutic regimen. 3-year RFS for patients receiving second-line adjuvant chemotherapy was 70.2%, and that for patients receiving the same chemotherapeutic regimen as the preoperative concurrent regimen was 56.7%. Thus, changing the adjuvant chemotherapy regimen did not affect the 3-year RFS of patients with yp Stage Ⅲ disease (Figure 2). Nevertheless the hazards ratio was more favor-able when a 2nd-line regimen was delivered. The risk of recurrence for patients with yp Stage Ⅲ disease who received second-line chemotherapy was 21% lower than that for patients receiving first-line chemotherapy (HR = 0.79, 95%CI: 0.39-1.63; $P = 0.53$; Table 2).

DISCUSSION

In the present study we stratified 5-year RFS according to the final pathologic stage in patients with rectal cancer treated by PCRT followed by radical resection. The AJCC TNM staging system is widely used for prognosis and for predicting the risk of recurrence in rectal cancer patients after surgical resection. However, the TNM staging system was originally based on pathologic findings in patients who did not receive neoadjuvant therapy prior to surgical resection. At present, the applicability and prognostic significance of the TNM staging system for patients that have undergone PCRT is not clear.

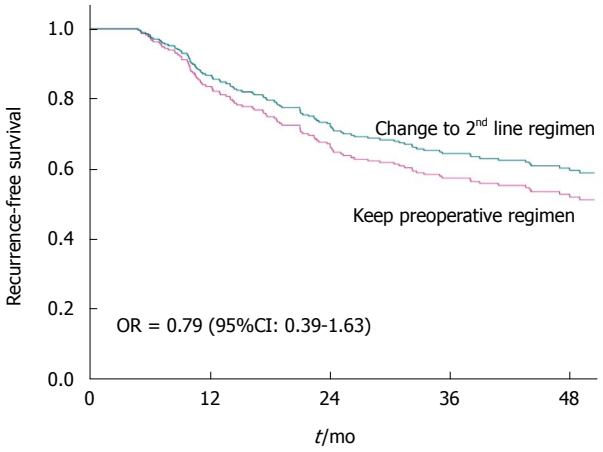

Figure 2 Recurrence-free survival of patients with yp Stage Ⅲ disease treated with different adjuvant chemotherapy regimens. Patients with yp Stage Ⅲ that received a different second-line chemotherapy regimen had longer recurrence-free survival than patients receiving a regimen that was the same as the preoperative concurrent regimen.

Some studies have found that patients showing a good response after PCRT have a more favorable prognosis, even in those patients with initially clinically node-positive disease[7-9,15,18]. On the other hand, patients with persistent nodal disease after chemoradiation have a poorer prognosis[7-9]. Thus, it is important to ascertain whether the use of postoperative chemotherapy should be decided by clinical stage, or by the definitive pathological surgical stage (ypTNM) following chemoradiotherapy.

The risk of recurrence is high for patients with clinical stage Ⅱ or Ⅲ rectal cancer; however, theoretically at least, the risk is not influenced by PCRT because the latter is a local treatment. However, data from this and other studies suggest that the risk of distant and local failure is, in fact, closely associated with the final pathologic stage. Typically a full course of adjuvant chemotherapy is recommended, regardless of the final pathologic stage. There are several reasons for this: preoperative chemotherapy uses a radiosensitizing agent rather than a definitive chemotherapy drug; the seminal randomized trials conducted for PCRT therapy included the use of routine adjuvant chemotherapy; and there may be a presumption that pathological stage is an unreliable prognostic indicator in patients treated with chemoradiotherapy. However, several studies show that pathological stage is in fact a reliable prognostic indicator, and that it may be more accurate than the preoperative clinical stage[7,18-20].

We found that, based on the pathologic stage, 5-year RFS in pathologic stage Ⅱ and Ⅲ was lower for the PCRT patients than for the No-PCRT patients. This suggests that the adjuvant chemotherapy for patients with pathologically-proven metastatic lymph nodes after PCRT should be different from that for No-PCRT patients. The authors of the EORTC 22921 study reported benefits of adjuvant chemotherapy for the subgroup showing down-staging after PCRT[21]. They proposed that only those patients achieving a pCR, or those that were down-staged to ypT1-2 after preoperative radiation, would benefit from adjuvant chemotherapy; those with residual ypT3-4 disease would not[21]. The study suggested that adjuvant chemotherapy had a beneficial effect when its administration was based on pathologic stage; however, the results for the ypT and ypN stages were analyzed separately. Adjuvant chemotherapy appeared to benefit patients that were down-staged (in terms of ypT stage) but had no effect according to ypN status[21]. Other studies did not confirm these results, particularly regarding the effect of adjuvant chemotherapy on patients that achieved pCR[15,18]. In view of the favorable outcomes for patients showing complete remission, it may be difficult to improve survival with adjuvant chemotherapy over and above that achieved without adjuvant chemotherapy.

In the present study we were not able to assess the benefits of adjuvant chemotherapy for patients showing complete remission because the number of such patients not receiving adjuvant chemotherapy was too small. However, we did examine the influence of adjuvant chemotherapeutic regimens on the RFS of the patients in the PCRT group with ypN+ disease (who had much poorer oncologic outcomes than those in the non-PCRT group). Those patients in the PRCT group that received changed adjuvant chemotherapy had a more favorable outcome than those who did not, although the difference was not statistically significant.

We also examined the effect of changing the adjuvant chemotherapy regimen used to treat patients with ypT3-4 stage disease. We found that 3-year RFS was higher when second-line chemotherapy was provided, although the difference was smaller (76.5% for patients with an altered second-line regimen *vs* 69.6% for those with the same regimen as mentioned used preoperatively) than that observed for the ypN+ patients (70.2% for patients with an altered second-line regimen *vs* 56.7% for those with a same regimen). A previous study reported higher rates of relapse despite adjuvant chemotherapy in patients who did not respond to preoperative treatment, and suggested that FOLFOX (oxaliplatin plus FL) be used for high-risk patients[4].

The 5-year RFS for patients showing complete remission after PCRT was comparable to that for patients with tumors confined within the rectal mucosa, which can be successfully treated by endoscopic resection or local excision. Therefore, organ-preserving treatments may be useful for patients showing complete remission after PCRT. In the present study, however, 90.2% of the latter received adjuvant chemotherapy, and all underwent radical resection. Great care should be taken when adopting an organ-preserving strategy in clinical practice.

The present study has several limitations. First, it was retrospective in nature, which may cause a bias towards the identification of metastasis/recurrence. However, we chose RFS as the outcome measure as it is less likely to be subject to selection bias or to be confounded by other parameters. We also used multivariate regression

Table 2 Univariate and multivariate cox proportional hazards regression models of the clinical factors associated with recurrence-free survival in preoperative chemoradiotherapy patients with pathologically- proven metastatic lymph nodes

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Sex			0.41			
Male	1.00					
Female	0.85	0.57-1.25				
Age (yr)	1.00	0.98-1.02	0.89			
Lymphovascular invasion			0.54			
None	1.00					
Present	1.28	0.83-1.98				
Perineural invasion			0.01			0.02
None	1.00			1.00		
Present	1.89	1.22-2.95		1.82	1.15-2.87	
Sphincter preservation			0.01			0.93
No	1.00			1.00		
Yes	0.60	0.40-0.89		0.96	0.35-2.59	
Tumor grade			0.34			
G1, G2	1.00					
G3, G4	1.31	0.75-2.31				
Preoperative CEA			0.45			
Normal	1.00					
Increased	1.22	0.73-2.05				
Location of tumor			0.25			0.89
Mid-rectum	1.00					
Lower rectum	1.26	0.85-1.87		1.03	0.66-1.62	
Number of retrieved lymph node	0.97	0.95-1.01	0.14			
Adjuvant chemotherapy[1]			0.36			0.46
Same[1]	1.00			1.00		
Altered	0.72	0.35-1.47		0.76	0.36-1.58	

[1]Same: same regimen as that used for preoperative concurrent chemoradiotherapy. Altered: Second-line regimen was different from that used for preoperative chemotherapy; CEA: Carcinoembryonic antigen.

to adjust for other potential confounders. In addition, we included patients diagnosed as cT3-4 or N+ based only on MRI in order to compensate for selection bias because of the variable accuracy of imaging modalities in the local staging of rectal cancer.

Second, very few of the patients treated with PCRT followed by radical resection received an altered second-line adjuvant chemotherapy regimen. Because of this (and the retrospective nature of this study) it would be inappropriate to conclude that using the same adjuvant chemotherapy with concurrent preoperative chemotherapeutic regimen based on clinical stage conferred no survival benefit. In addition, oxaliplatin was used as the adjuvant chemotherapeutic regimen since 2007. The number of patients receiving oxaliplatin, along with the shorter follow-up times for these patients, may have affected the final oncologic outcomes.

In conclusion, the final pathologic stage of patients with advanced rectal cancer treated by PCRT can be used to predict oncologic outcome. Thus, we suggest that intensive adjuvant chemotherapy might be considered for patients showing much poorer outcomes than those who are not treated with PCRT. Further large-scale studies should be performed to examine the reliability of pathologic stage as a prognostic indicator and guideline for adjuvant treatment in patients with rectal cancer treated by PCRT based on pathologic stage. It will be important to establish a standard to compare prognoses and to conduct clinical trials with the hope of influencing prognosis.

COMMENTS

Background

Recurrence-risk stratification is necessary to make evidence for adjuvant treatment to reduce disease recurrence and improve survival. Pathologic stage has been used for this purpose. Although preoperative chemoradiotherapy (PCRT) is established as a standard treatment for locally advanced rectal cancer, method for risk-stratification which is useful for post-surgical clinical practice was not settled. In addition the authors did not have overview impression for prognosis for PCRT patients. To give overview impression for prognosis of PCRT, the present study used prognosis based on pathologic stage in patient who did not receive PCRT because it is already well known in setting of clinical practice.

Research frontiers

Risk of recurrence was well stratified based on pathologic stage in PCRT patients. In case of nodal metastasis after PCRT showed much worse prognosis than those with node metastasis without PCRT. In these cases, there was a tendency of improvement of recurrence-free survival when 2nd-line chemotherapy was given. Future investigation is required to decide on clinical suitability of pathologic stage in PCRT patients.

Innovations and breakthroughs

The current study shows not only the difference of prognosis according to pathologic stage in patients treated with PCRT, but also possibility as a standard for adjuvant treatment and measurement of results. The present study also gives impression of prognosis of PCRT patients according to pathologic stage which is familiar to clinicians.

Applications

The final pathologic stage of patients with advanced rectal cancer treated by PCRT can be used to predict oncologic outcome. Adjuvant treatment

and surveillance need to be given based on prognostic implication based on pathologic stage. Pathologic stage also would be used as a standard to compare results of treatment in future investigations.

Peer review

The authors presented the data of prognosis based on pathologic stage of locally advanced rectal cancer patients treated with PCRT. The present study showed potential role of pathologic stage as a standard for measurement of treatment outcome peculiarity, although prognostic implication of pathologic stage in PCRT patient were also reported in other similar articles. It would be more useful for clinical practice to evaluate treatment outcome or to give intensive adjuvant treatment in PCRT patients because benefit of each adjuvant treatment in PCRT patients has not been established yet.

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ORIGINAL ARTICLE

Retrospective Study

Clinicopathologic factors and molecular markers related to lymph node metastasis in early gastric cancer

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Abstract

AIM: To analyze predictive factors for lymph node metastasis in early gastric cancer.

METHODS: We analyzed 1104 patients with early gastric cancer (EGC) who underwent a gastrectomy with lymph-node dissection from May 2003 through July 2011. The clinicopathologic factors and molecular markers were assessed as predictors for lymph node metastasis. Molecular markers such as microsatellite instability, human mutL homolog 1, p53, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) were included. The χ^2 test and logistic regression analysis were used to determine clinicopathologic parameters.

RESULTS: Lymph node metastasis was observed in 104 (9.4%) of 1104 patients. Among 104 cases of lymph node positive patients, 24 patients (3.8%) were mucosal cancers and 80 patients (16.7%) were submucosal. According to histologic evaluation, the number of lymph node metastasis found was 4 (1.7%) for well differentiated tubular adenocarcinoma, 45 (11.3%) for moderately differentiated tubular adenocarcinoma, 36 (14.8%) for poorly differentiated tubular adenocarcinoma, and 19 (8.4%) for signet ring cell carcinoma. Of 690 EGC cases, 77 cases (11.2%) showed EGFR overexpression. HER2 overexpression was present in 110 cases (27.1%) of 406 EGC patients. With multivariate analysis, female gender (OR = 2.281, P = 0.009), presence of lymphovascular invasion (OR = 10.950, P < 0.0001), diameter ($\geqslant$ 20 mm, OR = 3.173, P = 0.01), and EGFR overexpression (OR = 2.185, P = 0.044) were independent risk factors for lymph node involvement.

CONCLUSION: Female gender, tumor size, lymphovascular invasion and EGFR overexpression were predictive risk factors for lymph node metastasis in EGC.

Key words: Receptor; Epidermal growth factor; Stomach neoplasms; Carcinoma; Neoplasm metastasis; Lymph node

Core tip: We analyzed the factors related lymph node metastasis in early gastric cancer. The factors were not only clinicopathologic finding but also molecular biomarkers. It is unique because of the first study about biomarker related with metastatic lymph node in early gastric cancer.

Jin EH, Lee DH, Jung SA, Shim KN, Seo JY, Kim N, Shin CM, Yoon H, Jung HC. Clinicopathologic factors and molecular markers related to lymph node metastasis in early gastric cancer. *World J Gastroenterol* 2015; 21(2): 571-577 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/571.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.571

INTRODUCTION

Early gastric cancer (EGC) is defined as cancer invasion confined to the mucosa or submucosa, irrespective of lymph node metastasis[1,2]. Radical gastrectomy with lymph node dissection is the procedure of choice for EGC. Because the prognosis of patients with EGC has improved, the treatment strategies for EGC now include the improvement of quality of life.

Recently, endoscopic mucosal resection (EMR) has been widely accepted as an alternative treatment to open surgery for early gastric cancer without lymph node metastasis (LNM)[3,4]. EMR preserves gastric function and maintains a high quality of life, while extensive surgery carries a significant risk of morbidity and mortality. However, the indications for EMR are limited to EGC with elevated lesions < 2 cm in diameter and differentiated mucosal cancer without ulceration[4]. An endoscopic technique has included endoscopic submucosal dissection (ESD) that can be used to remove a larger amount of tumor *en bloc* with a negative safety margin[5]. In order to apply endoscopic techniques such as EMR/ESD to treat EGC, the absence of lymph node metastasis must be confirmed. Identifying patients at high risk for LNM is important for the application of a minimally-invasive endoscopic technique.

Several molecular markers have been reported to be useful predictors for prognosis of gastric cancer. Microsatellite instability (MSI) is a form of genomic instability that is associated with defective DNA mismatch repair in tumors[6]. In gastric cancer, the frequency of a microsatellite instability-high (MSI-H) phenotype was reported to range from 8.2% to 37%[7,8]. Several studies have shown that MSI in gastric cancers was an independent predictive factor of lower LNM and improved survival[9]. In addition, MSI was directly associated with the function of a mismatch repair gene such as human mutL homolog 1 (hMLH1)[10]. A study showed that hMLH1 methylation plays a probable role in the advanced stages of tumor progression[11]. In addition, mutation of the *p53* gene is one of the most frequent genetic abnormalities associated with gastric cancer; it is associated with lymph node metastasis in EGC[12]. Moreover, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) overexpression were associated with disease recurrence and poor prognosis in gastric cancer patients[13,14]. Thus, the aim of this study was to identify the clinicopathologic factors and molecular markers related lymph node metastasis and to identify high risk patients for minimal invasive therapy.

MATERIALS AND METHODS

Patients

A retrospective review identified 1104 patients with EGC who underwent a radical gastrectomy with regional lymph-node dissection from May 2003 through July 2011 at Seoul National University Bundang Hospital (Seoul, South Korea). This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (IRB No. B-1308-214-101). Patients were excluded if they had a recurrence or multifocal gastric cancer. The Histologic type was classified according to the World Health Organization classification for gastric cancer. Undifferentiated gastric carcinoma included poorly differentiated tubular adenocarcinoma (PD) and signet ring cell carcinoma (SRC). Well-differentiated (WD) and moderately-differentiated tubular adenocarcinoma (MD) were classified as the differentiated type. The relationship between the various clinicopathologic factors, molecular markers and lymph node metastasis were analyzed to identify the risk factors that were predictive of lymph node metastasis. These factors included: age (< 60 years or ≥ 60 years), sex, tumor size, location (upper third, middle third, or lower third), gross type of lesion (elevated, depressed, flat, or mixed), depth of invasion, lymphatic-vascular involvement, and histological type. Molecular markers such as MSI, hMLH1, p53, EGFR and HER2 were analyzed.

The Japanese classification of gastric carcinoma was used to designate the gross type of tumor: type Ⅰ (protruded), type Ⅱa (superficial elevated), type Ⅱb (flat), type Ⅱc (superficial depressed), and type Ⅲ (excavated)[15]. Type Ⅰ, type Ⅱa, and a combination of these two types with Ⅱb were classified as the elevated type. Type Ⅱb was defined as a flat type. Type Ⅱc and Ⅲ lesions, as well as the combined lesions, were defined as the depressed type. Both the elevated and depressed types, such as type Ⅱa and Ⅱc, were classified as mixed types.

Microsatellite instability analysis

DNA was obtained from formalin-fixed, paraffin-embedded surgical sections. DNA was extracted from harvested tumor cells by standard proteinase-K digestion and phenol/chloroform extraction. Normal DNA was extracted from the surrounding normal tissue. Five microsatellite markers originally recommended by a NCI

Table 1 Baseline characteristics of patients with early gastric cancer (n = 1104)

Characteristics	Value
Age (yr)	
< 60	546 (49.5)
⩾ 60	558 (50.5)
mean ± SD	58.49 ± 11.63
Gender	
Male	709 (64.2)
Female	395 (35.8)
Size of tumor (mm)	
< 20 mm	397 (34.3)
⩾ 20 mm	725 (65.7)
mean ± SD	27.8 ± 17.8
Location	
Upper third	125 (11.3)
Middle third	325 (29.4)
Lower third	654 (59.2)
Macroscopic type	
Elevated (Ⅰ, Ⅱa, Ⅰ + Ⅱa, Ⅱa + Ⅱb)	86 (7.8)
Flat (Ⅱb)	81 (7.3)
Depressed (Ⅱc, Ⅲ, Ⅱb + Ⅲ)	815 (73.8)
Mixed	122 (11.1)
Depth of invasion	
Mucosa	625 (56.6)
Submucosa	
Sm 1	150 (13.6)
Sm 2	157 (14.2)
Sm 3	172 (15.6)
Ulcer	
Absent	958 (86.8)
Present	146 (13.2)
Lymphovascular invasion	
Absent	955 (86.5)
Present	149 (13.5)
Histological type	
Well differentiated	236 (21.4)
Moderate differentiated	398 (36.1)
Poorly differentiated	243 (22.0)
Signet ring cell	227 (20.6)
Lymph-node metastasis	
Negative	1000 (90.6)
Positive	104 (9.4)

Data are expressed as absolute numbers (percentage) or mean ± SD. Sm1: Upper third; Sm2: Middle third; Sm3: Lower third.

workshop on MSI (BAT-25, BAT-26, D2S123, D5S346 and D17S250) were used to analyze paired normal and tumor DNA for MSI. According to the guidelines of the international workshop of NCI, tumors were classified as MSI-H when at least 2 of the 5 markers displayed novel bands, MSI-low (MSI-L) when additional alleles were found with one of the five markers, and microsatellite stable (MSS) when all microsatellite markers examined displayed identical patterns in both tumor and normal tissue.

Immunohistochemistry

Core tissue biopsy specimens (2 mm in greatest dimension) were obtained from individual paraffin-embedded tumors (donor blocks) and arranged in new recipient blocks (tissue microarray blocks), using a trephine apparatus (Superbiochips Laboratories, Seoul, South Korea). Three separate core samples per tumor were obtained to counter the effects of tumor heterogeneity. Sections (4 mm) were cut from each tissue microarray block, deparaffinized, and dehydrated. Immunohistochemical staining for hMLH1, p53, EGFR, HER-2 was performed as previously described[16,17]. Immunohistochemical expression of HER-2 was scored using DAKO-Hercep Test kits as follows: score 0, no membrane staining at all or membrane staining in < 10% of tumor cells; score 1+, faint/barely perceptible partial membrane staining in > 10% of tumor cells; score 2+, weak to moderate staining of entire membrane in > 10% of tumor cells; and score 3+, strong staining of entire membrane in > 10% of tumor cells. Scores of 0 and 1+ were considered negative for HER-2 overexpression, and scores of 2+ and 3+ were considered positive. EGFR immunopositivity was scored by using the instructions supplied with the EGFR PharmDx kits; scores of 2+ and 3+ indicated overexpression.

Statistical analysis

To identify the predictive factors of lymph node metastasis, the data were analyzed by using Pearson's χ^2 test and an unpaired Student's t-test. Multivariate logistic regression analysis was then performed to evaluate the risk factors for LNM. $P < 0.05$ was considered to be statistically significant. Statistical calculations were performed using IBM SPSS (version 19).

RESULTS

Of the 1104 patients with EGC evaluation, the mean age was 58.5 years (range: 25-86 years). This study included 709 men and 395 women. The mean tumor size was 27.8 mm. Mucosal cancers were 625 (56.6%) and submucosal cancers were 479 (43.4%). According histologic classification, WD was 236 (21.4%), MD was 398 (36.1%), PD was 243 (22.0%), and SRCC was 227 (20.6%). In 104 of 1104 (9.4%) patients, pathologic specimens contained LNM (Table 1).

With molecular marker analysis, 909 (90.1%) of 1,009 EGCs showed MSS. MSI-L was observed in 3.1% and MSI-H was observed in 6.8% of EGCs. Of 764 patients, 48 (6.3%) were deemed to have loss of hMLH1, while 716 (93.7%) had expression of hMLH1. Loss of p53 was seen in 651 (62.2%) of 716 patients. Of 690 EGC cases, 77 cases (11.2%) showed EGFR overexpression. In addition, HER2 overexpression was found in 110 cases (27.1%) of 406 EGC patients (Table 2).

The respective rate of LNM was 3.8% among lesions confined to the mucosa and 16.7% among those infiltrating the submucosa (sm1 cancer, 7.3%; sm2 cancer, 21.6%; sm3 cancer, 20.3%). According to histologic evaluation, the number of lymph node metastasis found was 4 (1.7%) for WD cancer, 45 (11.3%) for MD cancer, 36 (14.8%) for PD cancer, and 19 (8.4%) for SRC cancer. Lymph node metastasis was more frequent in MD than SRC cancers.

With univariate analysis, lymph node metastasis was

Table 2 Molecular markers of patients with early gastric cancer *n* (%)

Molecular markers	Value
Microsatellite instability	
MSS	909 (90.1)
MSI-L	31 (3.1)
MSI-H	69 (6.8)
hMLH1	
Loss	48 (6.3)
Expression	716 (93.7)
p53	
Negative	651 (62.2)
Positive	396 (37.8)
EGFR overexpression	
Negative	613 (88.8)
Positive	77 (11.2)
HER2 overexpression	
Negative	296 (72.9)
Positive	110 (27.1)

MSS: Microsatellite stable; MSI-L: Microsatellite instability-low; MSI-H: Microsatellite instability-high; hMLH1: Human mutL homolog 1; EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor 2.

Table 3 Univariate analysis of potential risk factors for lymph node metastasis *n* (%)

Factor for lymph node metastasis	Presence (*n* = 104)	Absence (*n* = 1000)	*P* value
Age (yr)			0.049
< 60	61 (58.7)	485 (48.5)	
⩾ 60	43 (41.3)	515 (51.5)	
Gender			0.003
Male	53 (51.0)	656 (65.6)	
Female	51 (49.0)	344 (34.4)	
Size of tumor (mm)			< 0.0001
< 20 mm	9 (8.7)	370 (37.0)	
⩾ 20 mm	95 (91.3)	630 (63.0)	
Location			0.389
Upper third	9 (8.7)	116 (11.6)	
Middle third	36 (34.6)	289 (28.9)	
Lower third	59 (56.7)	595 (59.5)	
Macroscopic type			< 0.0001
Elevated	7 (6.7)	78 (7.8)	
Flat	1 (1.0)	80 (8)	
Depressed	72 (69.2)	743 (74.3)	
Mixed	24 (23.1)	98 (9.8)	
Depth of invasion			< 0.0001
Mucosa	24 (23.1)	601 (60.1)	
Submucosa	80 (77.0)	399 (39.9)	
Sm1	11 (10.6)	139 (13.9)	
Sm2	34 (32.7)	123 (12.3)	
Sm3	35 (33.7)	137 (13.7)	
Ulceration			0.222
Absent	86 (9.0)	872 (91.0)	
Present	18 (12.1)	128 (85.9)	
Lymphovascular invasion			< 0.0001
Absent	44 (42.3)	911 (91.1)	
Present	60 (57.7)	89 (8.9)	
Histological type			< 0.0001
Well differentiated	4 (3.8)	232 (23.2)	
Moderate differentiated	45 (43.3)	352 (35.2)	
Poorly differentiated	36 (34.6)	208 (20.8)	
Signet ring cell	19 (18.3)	208 (20.8)	

Sm1: Upper third; Sm2: Middle third; Sm3: Lower third.

associated with age (⩾ 60 years), female gender, tumor size (⩾ 20 mm), macroscopic type, depth of invasion, lymphovascular invasion, and histological type (Table 3). Among molecular markers, EGFR overexpression was significantly associated with lymph node metastasis in early gastric cancer (Table 4). Of these factors, female gender, large tumor size (⩾ 20 mm), lymphovascular invasion, and EGFR overexpression were independently associated with lymph node metastasis by multivariate logistic regression analysis (Table 5).

DISCUSSION

Gastric cancer is the second leading cause of cancer-related deaths worldwide[18], and the highest mortality rates of AGC have been reported in East Asia including Japan and South Korea[19,20]. In contrast, EGC has a good prognosis with surgical treatment[21]. In South Korea, the proportion of EGC increased to 47.4% of all diagnosed gastric cancers in 2004[22]. This was attributed to widely-performed upper gastrointestinal endoscopy screening programs. Because the prognosis of patients with EGC has improved with radical gastrectomy, the treatment strategies for EGC now include the improvement of quality of life. Endoscopic resection such as EMR/ESD can be applied to EGC without lymph node metastasis instead of a radical gastrectomy[3,4].

Preoperative evaluation of for lymph node metastasis is the most important consideration, when deciding on a treatment strategy for EGC[23]. A number of researchers have attempted to identify factors predictive of LNM in EGC. The size of the primary tumor, histologic type, lymphatic or venous invasion, and depth of invasion are known to be associated with regional lymph node metastases in EGC[24-27]. In addition, multi-detector computerized tomography (MDCT) and/or endoscopic ultrasound (EUS) were generally employed to detect metastatic lymphadenopathy. However, the overall diagnostic accuracy of MDCT imaging for LNM in EGC has been reported to range from 37% to 70%, whereas that of EUS was reported to range from 39% to 90%[28-30]. Reported sensitivity and specificity of EUS to detect LNM in gastric cancer varies widely: sensitivity from 59.5% to 97.2% and specificity from 40.0% to 100%[1]. Using MDCT, studies showed a sensitivity of 84.2% and a specificity of 84.0%[1]. Preoperational accuracy of LNM staging using EUS or CT was inadequate for the prediction of the pathological N stage in order to determine the treatment plan.

Not only clinicopathologic factors but also molecular markers can be predictors for lymph node metastasis in gastric cancer patients[13,14]. The human epidermal growth factor receptor (HER) consists of four transmembrane tyrosine kinase receptors, which have a similar structure, are named ErbB1 (HER1, also known as EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4)[31].

Table 4 Univariate analysis of predictive molecular markers for lymph node metastasis *n* (%)

Lymph node metastasis	Presence	Absence	*P* value
Microsatellite instability			0.412
MSS	89 (90.8)	820 (90.0)	
MSI-L	1 (1.0)	30 (3.3)	
MSI-H	8 (8.2)	61 (6.7)	
hMLH1			0.703
Negative	5 (7.4)	43 (6.2)	
Positive	63 (92.6)	653 (93.8)	
p53			0.773
Negative	59 (60.8)	592 (62.3)	
Positive	38 (39.2)	358 (37.7)	
EGFR overexpression			0.001
Negative	55 (77.5)	558 (90.1)	
Positive	16 (22.5)	61 (9.9)	
HER2 overexpression			0.084
Negative	33 (84.6)	263 (71.7)	
Positive	6 (15.4)	104 (28.3)	

MSS: Microsatellite stable; MSI-L: Microsatellite instability-low; MSI-H: Microsatellite instability-high; hMLH1: Human mutL homolog 1; EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor 2.

Table 5 Multivariate analysis of potential risk characteristics for lymph node metastasis

Characteristics	Odds ratio	95%CI	*P* value
Gender (female)	2.281	1.228-4.235	0.009
Lymphovascular invasion	10.950	5.418-22.134	< 0.0001
Diameter (≥ 20 mm)	3.173	1.324-7.603	0.010
EGFR	2.185	1.020-4.683	0.044

EGFR: Epidermal growth factor receptor.

Alterations in the expression of receptor tyrosine kinases pathways including EGFR, HER2 were proven to be critical factors for cancer cell survival[32]. EGFR expression correlated with disease recurrence and poorer survival in gastric cancer patients[13,14]. Furthermore, HER2 has predictive ability for estimating overall survival in gastric cancer patients and may be useful for determining their prognosis[14]. However, EGFR positivity, but not HER2 positivity, was associated with poor patient outcomes after a curative resection of stage Ⅱ/Ⅲ gastric cancer[33]. In our study, EGFR overexpression was an independent risk factor for lymph node metastasis in EGC patients. However, HER2 overexpression was not associated with lymph node metastasis. Previous studies have reported EGFR or HER2 overexpression in gastric cancer regardless of stage. Only a handful of studies were limited to early gastric cancer for EGFR or HER2 overexpression.

In this study, the clinicopathologic risk factors for lymph node metastasis were found to be female gender, the presence of lymph-vascular involvement, and tumor size > 2 cm. Lymph-vascular involvement and tumor size were consistent to those reported by previous studies. Interestingly, female gender was an independent predictive factor for LNM; this was a unique finding compared to a previous report. Male to female gender ratio was 1:1.08 among young patients (age < 40 years) and 2.5:1 in older patients (age > 40 years)[34]. Age-standardized and cumulative incidence rates of gastric cancer in males are approximately double those of females. This predominance of gastric cancer in males is related to a 10-to-15 year delay in female gastric cancer. The prevalence of gastric cancer in females is similar to that of males only after menopause[35]. This finding suggested that sex hormones (estrogens) protect woman from gastric cancer. In previous studies in South Korea, the incidence of lymph node metastasis in female EGC was higher than in male EGC and female gender is a predictive risk factor for lymph node metastasis[36,37]. However, this gender difference of lymph node metastasis in EGC was not shown in other populations. It is extremely difficult to generalize risk factors in all populations.

Some studies have reported a lower rate of LNM and better prognosis in EGC with SRC histology than cancer with PD[38,39]. Previous studies have reported a rate of LNM with SRC histology to range from 5.7% to 15%[23,38,40]. Our study found that the rate of LNM with SRC histology was lower than PD cancer and even MD (18.3% *vs* 34.6% and 18.3% *vs* 43.3%). However, the frequency of LNM in mucosal cancer with SRC histology was much higher than mucosal cancer with differentiated histology (0.0% in WD, 2.9% in MD, 10.6% in PD, and 9.6% in SRC). Based on our study, mucosal EGC with SRC histology still had a higher risk of LNM than differentiated EGC. We suggest that the application of EMR/ESD in EGC with SRC was inadequate (Table 6).

This study had some limitations. First, it was a retrospective study based on medical records in a single center. Because of its retrospective nature, we could not collect additional data such as family history, comorbidity, or life style. Second, we analyzed pathologic findings based on postoperative examination of the resected specimen. At the time of endoscopy, the endoscopist subjectively estimated tumor size and reported gross findings and the presence of ulceration; this may have caused a discrepancy between endoscopic findings and pathologic findings. Considering that the preoperative clinical decision was made by endoscopic findings, it may be difficult to apply our pathologic characteristics to determine treatment plans. However, endoscopic resection criteria including tumor size, presence of ulceration and gross finding were based on pathologic evaluation of a surgical specimen that was fixed in formalin[41]. In addition, endoscopic findings had an inter-observer variability. Third, not all surgical specimens underwent immunohistochemical staining. Finally, there is the problem of selection bias. To perform immunohistochemical staining on all the postoperative specimens in EGC is not cost effective. However, EGFR overexpression correlated with LNM and a poorer prognosis; therefore, EGFR targeted therapy may be considered as adjuvant therapy postoperatively for high risk

Table 6 Lymph node metastasis by depth of invasion and histological type *n* (%)

	Lymph node metastasis		
	Presence (*n* = 104)	Absence (*n* = 1000)	Total
Well differentiated			
Mucosa	0 (0.0)	176 (17.6)	176 (0.0)
Submucosa	4 (3.8)	56 (5.6)	60 (6.7)
Moderate differentiated			
Mucosa	3 (0.0)	165 (1.8)	168 (1.8)
Submucosa	42 (40.4)	187 (18.7)	229 (18.3)
Poorly differentiated			
Mucosa	11 (10.6)	112 (11.2)	123 (8.9)
Submucosa	25 (24.0)	96 (9.6)	121 (20.7)
Signet ring cell			
Mucosa	10 (9.6)	147 (14.7)	157 (6.4)
Submucosa	9 (8.7)	61 (6.1)	70 (12.9)

patients with lymph node metastasis in EGC. Despite of these limitations, our study has significance because we analyzed not only clinicopathologic factors but also molecular markers for a high risk of LNM in EGC patients.

Female gender, tumor size, and lymphovascular invasion were predictive risk factors for LNM in EGC. In addition, EGFR overexpression was identified as an independent prognostic factor with multivariate analysis; thus, suggesting that EGFR overexpression is likely to be one of the potential risk factor for LNM in EGC.

COMMENTS

Background

Endoscopic resection can be an alternative treatment to a radical gastrectomy for early gastric cancer without lymph node metastasis. The possible presence of lymph node metastasis is critical for the selection of the appropriate treatment strategy for early gastric cancer.

Research frontiers

This study to determine the predictive factors for lymph node metastasis in early gastric cancer (EGC). This is significant because the first research showed the biomarkers were related with lymph node metastasis in early gastric cancer.

Innovations and breakthroughs

In this study, epidermal growth factor receptor (EGFR) overexpression is one of the potential risk factors for lymph node metastasis in EGC.

Applications

The results suggest that patients who had EGFR overexpression in EGC were considered as high risk group for lymph node metastasis. Physicians pay attention to decide the treatment strategy.

Terminology

Microsatellite instability is the condition of genetic hypermutability that results from impaired DNA mismatch repair. The EGFR is the cell-surface receptor for members of the epidermal growth factor-family of extracellular protein ligands. Human epidermal growth factor receptor 2 is a member of the EGFR/ERBB family.

Peer review

This study analyzed 1104 patients with early gastric cancer who underwent a gastrectomy with lymph-node dissection. The goal was to assess predictive factors for lymph node metastasis in early gastric cancer. This is a general look at a specific tumor work up. The data suggest that EGFR overexpression is likely to be one of the potential risk factors for lymph node metastasis in EGC. This information may be value in helping the management of these subjects.

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ORIGINAL ARTICLE

Retrospective Study

Submucosal tunneling and endoscopic resection of submucosal tumors at the esophagogastric junction

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Abstract

AIM: To evaluate the safety and efficacy of submucosal tunneling and endoscopic resection (STER) for treating submucosal tumors (SMTs).

METHODS: Between August 2012 and October 2013, 21 patients with SMTs originating from the muscularis propria (MP) layer at the esophagogastric junction were treated by STER of their tumors. Key steps of the procedure include: (1) mucosal incision: a 2-cm longitudinal mucosal incision was made 5 cm proximal to the tumor; (2) submucosal tunneling: a submucosal tunnel was created 5 cm proximal to and 1 to 2 cm distal to the tumor; (3) tumor resection: the SMT was resected under direct endoscopic viewing; (4) hemostasis: while finishing the tumor resection, careful hemostasis of the MP defect and the tunnel was performed; and (5) mucosal closure: the mucosal incision site was closed by using hemostatic clips. During the operation, equipment used included a cap-fitted endoscope, an insulated-tip knife, a hook knife, hemostatic forceps, an injection needle, a snare, an endoclip, and a high-frequency generator. Carbon dioxide (CO_2) insufflation was achieved by using a CO_2 insufflator.

RESULTS: The median age of the patients was 46.2 years (range, 35-59 years), and the majority were male (18 male *vs* 3 female). Complete resection rate was 100% (21/21). Eighteen lesions were resected *en bloc*. Mean tumor size was 23 mm (range, 10-40 mm), and mean procedure time was 62.9 min (range, 45-90 min). Pathological diagnosis of these tumors included leiomyoma (15 out of 21) and gastrointestinal stromal tumor (6 out of 21). Full-thickness MP resection was performed in 9 of 21 patients (42.9%), with mediastinal and subcutaneous emphysema occurring in all nine. At the completion of the procedure, all patients received closure of the incision with hemoclips. One patient required percutaneous drainage. The remaining 20

patients required no further endoscopic or surgical intervention. There were no incidents of massive or delayed bleeding. The median follow-up period after the procedure was 6 mo (range, 2-14 mo). During follow-up, no patients were found to have residual or recurrent tumor or esophageal stricture.

CONCLUSION: STER is safe, effective and feasible, which provides accurate histopathologic evaluation and curative treatment for SMTs originating from the MP layer at the esophagogastric junction.

Key words: Submucosal tunneling and endoscopic resection; Esophagogastric junction; Subepithelial tumor; Muscularis propria layer; Submucosal tunneling

Core tip: Submucosal tunneling and endoscopic resection (STER) has emerged as a new technique for resecting upper gastrointestinal subepithelial tumors (SETs). This new endoscopic technique has advantages over conventional endoscopic muscularis excavation in terms of maintaining the integrity of the digestive tract mucosa and submucosa, promoting rapid wound healing, and reducing the risk of pleural/abdominal infection. The present study was conducted to evaluate the safety and efficacy of STER for SETs at the esophagogastric junction originating from the muscularis propria layer.

Zhou DJ, Dai ZB, Wells MM, Yu DL, Zhang J, Zhang L. Submucosal tunneling and endoscopic resection of submucosal tumors at the esophagogastric junction. *World J Gastroenterol* 2015; 21(2): 578-583 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/578.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.578

INTRODUCTION

Most esophageal and gastric cardia subepithelial tumors (SETs) are benign, but the possibility of transformation to malignancy considerably influences the attitude towards the entire group, especially when SETs originate from the muscularis propria (MP) layer[1-3]. Asymptomatic gastrointestinal submucosal tumors (SMTs) which are < 3 cm could be followed by periodic endoscopy and/or EUS or resection[4]. Conventional endoscopic muscularis excavation of tumors originating from the MP layer has been reported to be feasible and safe. However, SETs at the esophagogastric junction are often irregular and lobulated. Therefore, conventional endoscopic muscularis excavation results in larger mucosal defects which are difficult to close. Moreover, perforation is the common complication when SETs originating from the MP layer are treated by endoscopic resection. Submucosal tunneling and endoscopic resection (STER) has emerged as a new technique for resecting upper gastrointestinal SETs[5-7]. This new endoscopic technique has advantages over endoscopic submucosal dissection (ESD) in terms of maintaining the integrity of the digestive tract mucosa and submucosa, promoting rapid wound healing, and reducing the risk of pleural/abdominal infection. The present study was conducted to evaluate the safety and efficacy of STER for SETs at the esophagogastric junction originating from the MP layer.

MATERIALS AND METHODS

Participants

The current study was a retrospective study conducted at a single center in China. The study protocol was approved by the hospital's Internal Review Board and Ethics Committee. Informed consent was obtained for all procedures. Between August 2012 and October 2013, 21 patients with SETs originating from the MP layer at the esophagogastric junction were treated by STER. Endoscopic ultrasound (EUS) using an echoendoscope (UM 240; Olympus Optical Co. Ltd., Tokyo, Japan) and esophageal air-insufflation CT were performed to determine the size, layer of origin, margin, interval growth pattern of SETs, and the anatomical features of the adjacent structures (Figure 1B).

STER procedure

Patients were sedated with intravenous propofol (2 mg/kg). Endotracheal intubation was performed for positive pressure ventilation. Endoscopic equipment and accessories were sterilized in a standard manner. The esophagus and stomach were lavaged with levofloxacin (0.6 g/200 mL). Equipment used included a cap-fitted endoscope (GIF-H260, D-201-11802; Olympus, Tokyo, Japan), an insulated-tip knife (KD-611L, IT2; Olympus), a hook knife (KD-620LR, Olympus), hemostatic forceps (FD-410LR, Olympus), an injection needle (NM-4L-1, Olympus), a snare (SD-9L-1, Olympus), an endoclip (HX-600-135, Olympus), and a high-frequency generator (ICC-200, Erbe, Tu¨bingen, Germany). Carbon dioxide (CO_2) insufflation was achieved by using a CO_2 insufflator (UCR; Olympus, Tokyo, Japan).

The STER procedure was performed as follows. The lesion and potential location of the submucosal tunnel were injected with methylene blue or indigo carmine. A fluid cushion was created 5 cm proximal to the SET by injecting several milliliters of a solution containing 100 mL saline, 2 mL indigo carmine, and 1 mL epinephrine. A 2 cm longitudinal mucosal incision was made and a submucosal tunnel between the submucosal and muscular layers was created. Endoscopic resection of the SET was then performed through the created tunnel. When the lesion was completely resected, it was removed with a snare or forceps. All visible blood vessels were coagulated with hot biopsy forceps or by argon plasma coagulation. The mucosal incision site was closed with hemoclips (Figure 1).

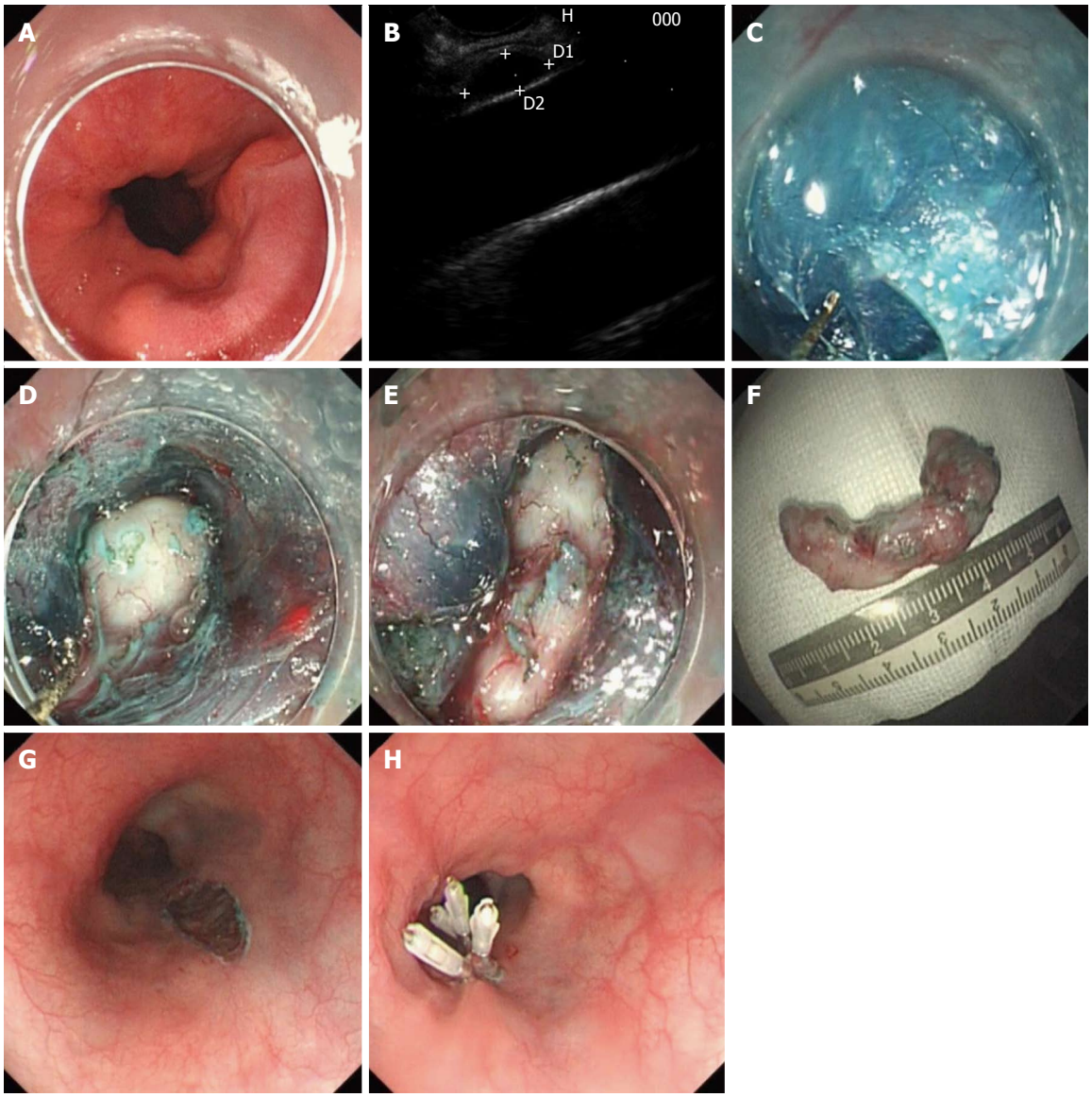

Figure 1 Submucosal tunneling and endoscopic resection of subepithelial tumors at the esophagogastric junction originating from the muscularis propria layer. A: Endoscopic view of subepithelial tumors; B: Endoscopic ultrasonographic evaluation of the same lesion; C: Submucosal tunnel to the lesion (with a hook knife); D: The exposed tumor; E: Annular growth of the tumor; F: The resected specimen; G: The mucosal entry incision; H: The closure of the mucosal entry incision (with several clips).

Definitions

During the procedure, any bleeding affecting the field of view and that could not be managed by endoscopic methods is considered a complication[8]. Delayed bleeding is defined as active post-procedure bleeding diagnosed by endoscopy[9]. Perforation is considered to be present if there is: endoscopic visualization of an extragastric structure during the procedure, subcutaneous emphysema, pneumothorax, pneumoperitoneum, or retroperitoneal gas with signs of peritonitis[10]. Resection is deemed incomplete when negative margins could not be established[10]. *En bloc* resection refers to resection in one piece. Procedure time is calculated from the beginning of the mucosal incision until the withdrawal of the endoscope.

Postoperative management

If there were no complications, an oral diet was restarted 1-2 d after the STER. Esomeprazole 40 mg twice daily (Astra Zeneca, Soderalje, Sweden) was administered intravenously during the patient's hospital stay, and then orally for another 4 wk. When pneumothorax, pneumoperitoneum, or subcutaneous emphysema occurred, patients were treated by gastrointestinal decompression, intravenous infusion of esomeprazole, intravenous antibiotics and suspension of oral diet for 2-3 d. When the patient had no abdominal pain and their vital signs were stabilized, they were progressed to a full fluid diet.

Pathological evaluation

Paraffin-embedded tissue sections were immunohistochemically

Table 1 Conditions of patients and effects of submucosal tunneling and endoscopic resection

Variable	Value
Age (yr)	46.2 yr (range, 35-59 yr)
Sex	
Male	18
Female	3
Tumor size (mm)	23 mm (range, 10-40 mm)
Pathological diagnosis	
Leiomyoma	15
GIST	6
Methods of resection	
Partial-thickness MP resection	12
Full-thickness MP resection	9
Complications	
Perforation	9
Bleeding	0
Delayed bleeding	0
Hospital stay (d)	4.3 (range, 3-7)
Follow-up period (mo)	6 (range, 2-14)

MP: Muscularis propria.

stained with DAKO antibodies (Dako Poland LTD, Gdynia, Poland). Tumors with positive staining for CD117 or DOG-1, as well as CD34 were considered diagnostic of a gastrointestinal stromal tumor. Tumors that were positive for smooth muscle actin and desmin were diagnosed as leiomyomas[7].

Follow-up

Surveillance endoscopy was performed to observe healing of the wound at 1, 3, and 6 mo. Surveillance EUS was performed at 3 mo.

RESULTS

The median age of the patients was 46.2 years (range, 35-59 years), and the male/female ratio was 6 (18 male *vs* 3 female). Complete resection rate was 100% (21/21). Eighteen lesions were resected *en bloc*. The mean size of tumors was 23 mm (range, 10-40 mm). Mean procedure time was 62.9 min (range, 45-90 min). Pathological diagnosis of these tumors included leiomyoma (15 out of 21) and gastrointestinal stromal tumor (6 out of 21). All patients had successful closure with hemoclips, which maintained the integrity of the digestive tract mucosa and submucosa. Full-thickness MP resection was performed in 9 of 21 patients (42.9%), with mediastinal and subcutaneous emphysema occurring in all nine. Nine patients receiving full-thickness MP resection were treated with anti-inflammatory therapy as well as fasting. Eight of nine patients had successful closure with hemoclips and required no further treatment. Mediastinal effusions were found in one patient, with obvious fever occurring. After treatment with anti-inflammatory therapy as well as percutaneous drainage, the patient was discharged 7 d later with normal temperature. There were no episodes of massive or delayed bleeding.

The median hospital stay after procedure was 4.3 d (range, 3-7 d). The median follow-up period after the procedure was 6 mo (range, 2-14 mo). No patients had residual or recurrent tumors detected and no patients had esophageal or gastric strictures during the follow-up period (Table 1).

DISCUSSION

Management of submucosal gastrointestinal tract lesions continues to be a challenging and controversial topic. Recent studies have reported that gastric SETs originating from the MP layer could be successfully removed by endoscopic full-thickness resection[11,12]. Management of SETs at the esophagogastric junction provides further difficulties. First, the esophagogastric junction is adjacent to the diaphragm, complicating the endoscopic resection with movement from breathing as well as esophageal peristalsis. Second, the shape of SETs of the esophagogastric junction is often irregular and lobulated. Moreover, SETs at the esophagogastric junction originating from the MP layer always grow annularly. Conventional endoscopic muscularis excavation causes large mucosal defects which are difficult to close and often result in strictures. Although conventional endoscopic submucosal excavation (ESE) and endoscopic full-thickness resection (EFR) are very effective methods for the removal of esophageal or cardial SETs originating from the MP, they are found to have difficulty in closing the mucosal incision site by using hemostatic clips and have the possibility of resulting in strictures after the formation of scar.

The submucosal tunneling technique was originally described by Sumiyama as an access for natural orifice transluminal endoscopic surgery[13]. This technique was later modified by Pasricha and Inoue, who started to use the submucosal tunnel as a working space for endoscopic myotomy in patients with achalasia[14,15]. Motivated and encouraged by this technique, Xu *et al*[6] used a similar submucosal tunnel as a working space for endoscopic muscularis dissection to resect esophageal or cardial SETs involving the MP layer, and named it STER.

Compared with the other conventional ESD techniques, the STER procedure differs in some aspects, although resection could be performed completely by both ESD and STER. First, a submucosal tunnel to the lesion is created between the submucosal and muscular layers. There is a certain distance between proximal mucosal incision and SETs, so the mucosal incision is regular and closed easily. Second, the gastroscope enters into the tunnel, providing an improved endoscopic view for the resection. Last but not least, when SETs originating from the MP layer are successfully removed, part of the MP layer is resected at the same time without harming the mucosa at the lesion, which reduces the risk of post-procedure stricture in theory.

In the present study, 21 patients with SETs at the esophagogastric junction originating from the MP layer underwent STER, and complete resection was achieved

in all 21 cases. Eighteen lesions were resected *en bloc*. Results have shown no significant difference in complete resection rate between ESE/EFR and STER. However, when endoscope enters submucosal tunnel, it has little effect on respiration during the procedure. What's more, a good field of view during the procedure contributes to more accurate operation.

Although conventional endoscopic muscularis excavation is a very effective method for the removal of esophageal or cardial SETs originating from the MP, perforation is frequently observed, especially in patients who have a full-thickness MP resection. In this study, considering that submucosal tumors were irregular and lobulated, and there was a close relationship between submucosal tumors and deep longitudinal muscle, 9 patients were treated by full-thickness MP resection. Of the 9 patients who had a full-thickness MP resection, the mucosal entry site was completely closed with endoscopic clips at the end of the procedure, preventing leakage of the gastrointestnal contents into the mediastinum or thoracoabdominal cavity. Although the mucosal layer becomes the only barrier between the gastrointestinal lumen and the visceral cavity, a similar situation is observed after the widely used Heller myotomy for achalasia, where a full-thickness myotomy is covered only by the esophageal mucosal layer[16]. All 21 patients recovered with conservative treatment without further endoscopic or surgical intervention. In this study, it was observed that all patients were diagnosed with mediastinum and subcutaneous emphysema. However, mediastinal effusions were found in only one patient, with obvious fever occurring. After treatment with anti-inflammatory therapy as well as percutaneous drainage, the patient was discharged 7 d later with normal temperature. No massive bleeding, delayed bleeding, or other severe complications occurred during or after the procedure. When it comes to recurrence after resection, it should be attached great importance to, especially for invasive tumors like gastrointestinal stromal tumors. In this study, no residual or recurrent tumors were detected and no strictures were identified during the follow-up period (median, 6 mo; range, 2-14 mo). Due to the limitation of short follow-up period, it requires further clinical observation. All in all, STER may provide a feasible, safe, and effective treatment strategy for providing accurate histopathologic evaluation and curative treatment for SETs at the esophagogastric junction originating from the MP layer.

COMMENTS

Background

Management of submucosal gastrointestinal tract lesions continues to be a challenging and controversial topic. Recent studies have reported that gastric subepithelial tumors (SETs) originating from the muscularis propria (MP) layer could be successfully removed by endoscopic full-thickness resection. Management of SETs at the esophagogastric junction provides further difficulties. First, the esophagogastric junction is adjacent to the diaphragm, complicating the endoscopic resection with movement from breathing as well as esophageal peristalsis. Second, the shape of SETs of the esophagogastric junction is often irregular and lobulated. Moreover, SETs at the esophagogastric junction originating from the MP layer always grow annularly. Conventional endoscopic muscularis excavation causes large mucosal defects which are difficult to close and often result in strictures. Therefore, the development of new technique for resecting upper gastrointestinal SETs remains attractive.

Research frontiers

The submucosal tunneling technique was originally described by Sumiyama as an access for natural orifice transluminal endoscopic surgery. This technique was later modified by Pasricha and Inoue, who started to use the submucosal tunnel as a working space for endoscopic myotomy in patients with achalasia. Motivated and encouraged by this technique, Xu used a similar submucosal tunnel as a working space for endoscopic muscularis dissection to resect esophageal or cardial SETs involving the MP layer, and named it submucosal tunneling and endoscopic resection (STER).

Innovations and breakthroughs

Conventional endoscopic muscularis excavation results in larger mucosal defects which are difficult to close. Moreover, perforation is the common complication when SETs originating from the MP layer are treated by endoscopic resection. However, STER has emerged as a new technique for resecting upper gastrointestinal SETs. This new endoscopic technique has advantages over endoscopic submucosal dissection (ESD) in terms of maintaining the integrity of the digestive tract mucosa and submucosa, promoting rapid wound healing, and reducing the risk of pleural/abdominal infection.

Applications

Submucosal tunneling and endoscopic resection is a safe, effective and feasible method, which provides accurate histopathologic evaluation and curative treatment for submucosal tumors (SMTs) originating from the MP layer at the esophagogastric junction.

Terminology

ESD is known as a kind of treatment modality for gastrointestinal epithelial lesions.

Peer review

This paper describes the whole procedure of STER for treating SMTs. The main thrust of the paper is that the authors aim to demonstrate its clinical efficacy for the treatment of SMTs at the esophagogastric junction. This paper is well written and reports an important study.

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ORIGINAL ARTICLE

Retrospective Study

Prophylaxis against hepatitis B virus recurrence after liver transplantation: A registry study

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Abstract

AIM: To evaluate the prophylactic efficacy of hepatitis B immunoglobulin (HBIG) in combination with different nucleos(t)ide analogues.

METHODS: A total of 5333 hepatitis B surface antigen-positive patients from the China Liver Transplant Registry database were enrolled between January 2000 and December 2009. Low-dose intramuscular (im) HBIG combined with one nucleos(t)ide analogue has been shown to be very cost-effective in recent reports. Hepatitis B virus (HBV) prophylactic outcomes were compared based on their posttransplant prophylactic protocols [group A (n = 4684): im HBIG plus lamivudine; group B (n = 491): im HBIG plus entecavir; group C (n = 158): im HBIG plus adefovir dipivoxil]. We compared the related baseline characteristics among the three groups, including the age, male sex, Meld score at the time of transplantation, Child-Pugh score at the time of transplantation, HCC, pre-transplantation hepatitis B e antigen positivity, pre-transplantation HBV deoxyribonucleic acid (HBV DNA) positivity, HBV DNA at the time of transplantation, pre-transplantation antiviral therapy, and the duration of antiviral therapy before transplantation of the patients. We also calculated the 1-, 3- and 5-year survival rates and HBV recurrence rates according to the different groups. All potential risk factors were analyzed using univariate and multivariate analyses.

RESULTS: The mean follow-up duration was 42.1 ± 30.3 mo. The 1-, 3- and 5-year survival rates were lower in group A than in groups B (86.2% *vs* 94.4%, 76.9% *vs* 86.6%, 73.7% *vs* 82.4%, respectively, $P < 0.001$) and C (86.2% *vs* 92.5%, 76.9% *vs* 73.7%, 87.0% *vs* 81.6%, respectively, $P < 0.001$). The 1-, 3- and 5-year posttransplant HBV recurrence rates were significantly higher in group A than in group B (1.7% *vs* 0.5%, 3.5% *vs* 1.5%, 4.7% *vs* 1.5%, respectively, $P = 0.023$). No significant difference existed between groups A and C and between groups B and C with respect to the 1-, 3- and 5-year HBV recurrence rates. Pretransplant hepatocellular carcinoma, high viral load and posttransplant prophylactic protocol (lamivudine and HBIG *vs* entecavir and HBIG) were associated with HBV recurrence.

CONCLUSION: Low-dose intramuscular HBIG in combination with a nucleos(t)ide analogue provides effective prophylaxis against posttransplant HBV recurrence, especially for HBIG plus entecavir.

Key words: Viral hepatitis; Recurrence; Hepatitis B immunoglobulin; Liver transplantation; Nucleos(t)ide analogue

Core tip: Little is known about which protocol has the optimal prophylactic effects against hepatitis B virus (HBV) recurrence. In this study, we used data from the China Liver Transplant Registry database to evaluate the long-term prophylactic efficacy of hepatitis B immunoglobulin (HBIG) in combination with different nucleos(t)ide analogues and determine the risk factors for HBV recurrence. This nationwide multicenter study demonstrated that low-dose intramuscular HBIG in combination with a nucleos(t)ide analogue provides effective prophylaxis against recurrent HBV infection posttransplantation at approximately 5% of the cost of conventional high-dose intravenous HBIG regimens. Among them, low-dose intramuscular HBIG combined with entecavir has better prophylactic efficacy than the combination of low-dose intramuscular HBIG and lamivudine.

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INTRODUCTION

Globally, chronic hepatitis B remains the leading cause of liver-related mortality and accounts for more than one million deaths *per annum*. Hepatitis B virus (HBV)-related liver diseases account for approximately 78% of all adult liver transplant recipients[1]. In selected patients with end-stage HBV-related liver diseases, liver transplantation (LT) offers a life-saving treatment with a 5-year survival rate of approximately 70%-80%. However, the main problem in hepatitis B surface antigen (HBsAg)-positive recipients is the risk of HBV recurrence posttransplantation, which may lead to rapid disease progression or even death[2,3].

Before the availability of antiviral prophylaxis, HBV-related liver disease was considered a relative contraindication for LT because of a high HBV recurrence rate (80%)[4]. In 1987, hepatitis B immunoglobulin (HBIG) became available and its long-term use reduced the 3-year actuarial risk of HBV reinfection from 74% to 36%[5]. However, HBIG monotherapy has several disadvantages, including high cost, inconvenient administration and adverse effects. Currently, HBIG monotherapy is seldom used for prophylaxis against HBV recurrence after LT. Lamivudine (LAM) was subsequently considered a potential prophylactic agent in LT because it is inexpensive and well tolerated. However, the initial enthusiasm was tempered by the realization that long-term LAM monotherapy is associated with drug resistance leading to increased HBV reinfection[6,7].

Compared with the monotherapy, combination therapy with LAM and high-dose intravenous (iv) HBIG has shown encouraging outcomes with an HBV recurrence rate of less than 10% in 1-2 years of follow-up[8]. However, the major limitation of this regimen is its high cost, and other factors, including inconvenient administration and unavailability of iv HBIG in some countries. In China, many centers accept the prophylactic protocol with LAM and low-dose intramuscular (im) HBIG due to the national conditions and unavailability of iv HBIG. With the introduction of new nucleos(t)ide analogues, such as adefovir dipivoxil (ADV), telbivudine and entecavir (ETV), some centers also chose the protocol with another nucleos(t)ide analogue and im HBIG to prevent HBV reinfection after LT.

Using data from the China Liver Transplant Registry database, the aim of this study was to evaluate the long-term prophylactic efficacy of HBIG in conjunction with different nucleos(t)ide analogues in China and identify the risk factors for posttransplant HBV recurrence.

MATERIALS AND METHODS

Patient cohort

Figure 1 shows the inclusion and exclusion criteria for the cohort from the China Liver Transplant Registry database (https://www.cltr.org/). A total of 13273 adult HBsAg-positive patients were initially enrolled between January 2000 and December 2009; however, 168 patients with suspect data or with oral antiviral drug resistance before LT were excluded. After excluding 7727 patients who had incomplete data for analysis or did not use the prophylactic protocol with low-dose im HBIG and one nucleos(t)ide analogue, 5378 patients remained. We excluded an additional 45 patients with low-dose im HBIG and telbivudine because of the small size sample. Finally, 5333 patients were included. The patients were divided into the following three groups based on the nucleos(t)ide analogues used for the prophylaxis protocol: group A (n = 4684), which consisted of patients with HBIG and LAM; group B (n = 491), which consisted of those with HBIG and ETV; and group C (n = 158), which consisted of those with HBIG and ADV. The patients were monitored until September 2012 or until they were deceased, and their medical records were retrospectively reviewed. Living and deceased donations were voluntary and altruistic in all cases, approved by Ethics Committee of West China Hospital of Sichuan University, and in accordance with the ethical guidelines

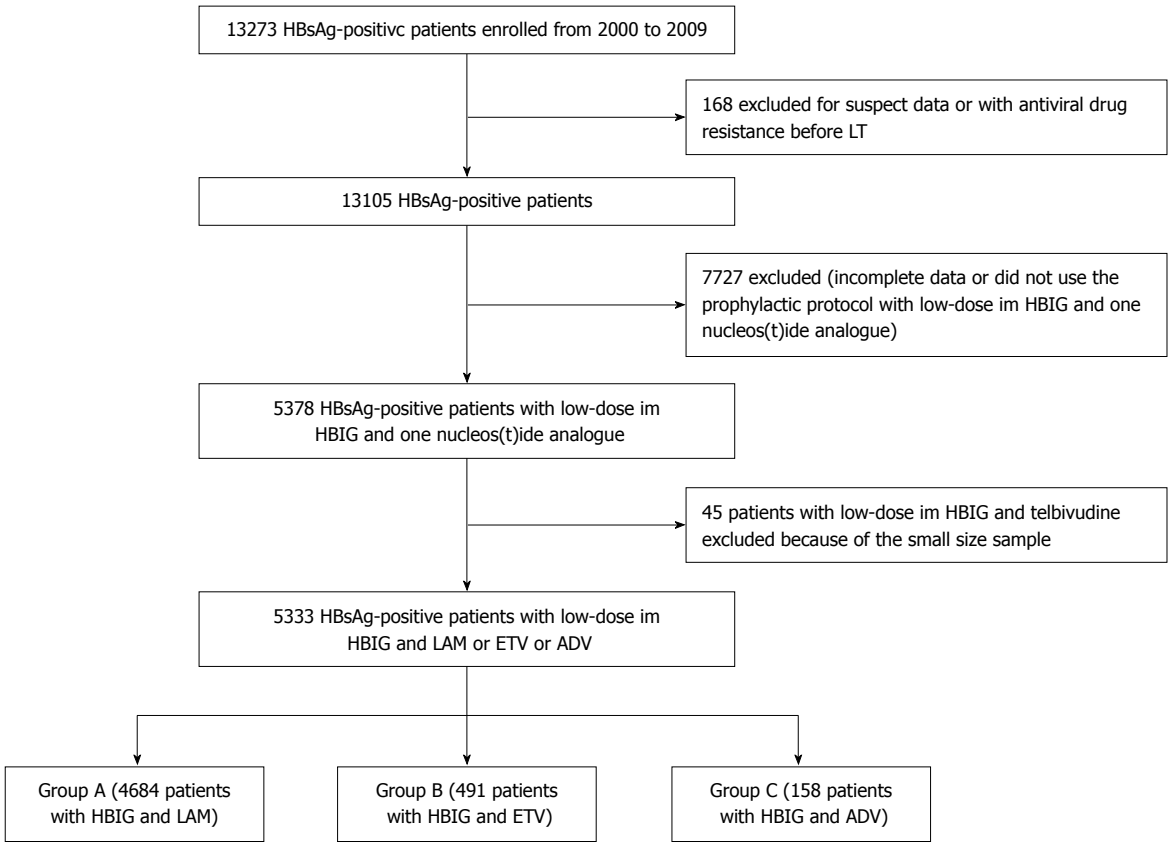

Figure 1 Flow of enrollment of study participants. HBsAg: Hepatitis B surface antigen; LT: Liver transplantation; im: Intramuscular; HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir dipivoxil.

of the Declaration of Helsinki. Written informed consent was given by participants for their clinical records to be used in this study.

HBV prophylaxis protocol

Prior to LT, patients with detectable serum HBV DNA received one nucleos(t)ide analogue daily, such as LAM, ETV or ADV, and the same nucleos(t)ide analogue was administered posttransplantation. HBIG was administered intramuscularly using a fixed dosing schedule, which consisted of 2000 IU of HBIG in the anhepatic phase, followed by 800 IU daily for the next 6 d, followed by weekly for 3 wk, and monthly thereafter.

Immunosuppression

Maintenance immunosuppression consisted of a triple-drug regimen that included tacrolimus or cyclosporine, mycophenolate and prednisone. Prednisone was generally discontinued within 3 to 6 mo after LT.

HBV evaluation

Prior to LT, viral markers including HBsAg, hepatitis B surface antibody (HBsAb), hepatitis B e antigen (HBeAg), hepatitis B e antibody, hepatitis B core antibody (HBcAb) and antibody to hepatitis C virus were routinely measured using standard commercial assays (Abbott Laboratories, Chicago, IL) as part of the Pre-LT workup for recipients and donors. Serum HBV DNA was determined using quantitative polymerase chain reaction method, with a limit of detection of 1000 copies/mL. After LT, liver function profiles were checked daily for the first week and then weekly for the first month, and monthly thereafter. Serum HBV markers were monitored weekly for the first month and monthly thereafter, and HBV DNA levels were evaluated monthly. HBV recurrence was defined as the reappearance of either HBsAg or HBV DNA in the serum. Liver biopsies were performed when clinically indicated by an elevation in serum liver enzyme levels.

Statistical analysis

SAS 9.2 statistical software was used to analyze the relevant data. Categorical data were presented as a number (percent) and compared using a χ^2 test. Continuous variables were expressed as mean ± SD, and analyzed using the Wilcoxon test. Survival curves and HBV recurrence were estimated using the Kaplan-Meier method and differences among ordered categories were determined by log-rank test. The Cox proportional hazards model was used to test potential predictors of HBV recurrence after LT. Univariate results were reported as hazard ratios with 95%CI. The variables reaching statistical significance ($P < 0.10$) by univariate analysis were then included for multivariate analysis with proportional hazard regression. $P < 0.05$ was considered statistically significant.

Table 1 Baseline characteristics of the recipients and their donors *n* (%)

	Group A	Group B	Group C	*P* value		
				A <-> B	A <-> C	B <-> C
Number of patients	4684	491	158	-	-	-
Age, mean ± SD (range) (yr)	48.2 ± 9.3 (19-76)	48.3 ± 9.5 (19-73)	48.4 ± 8 (26-71)	0.892	0.998	0.956
Male sex	4136 (88.3)	436 (88.8)	142 (89.9)	0.744	0.544	0.707
MELD score at LT, mean ± SD (range)	18.0 ± 9.5 (6-84)	17.6 ± 10.1 (6-65)	16.8 ± 9.2 (6-50)	0.205	0.250	0.847
Child-Pugh score at LT, mean ± SD (range)	8.9 ± 2.5 (5-15)	8.7 ± 2.8 (5-15)	8.7 ± 2.7 (5-14)	0.391	0.771	0.999
With HCC	2146 (45.8)	251 (51.1)	76 (48.1)	0.025	0.571	0.509
Pre-LT HBeAg positivity	1169 (25.0)	171 (34.8)	58 (36.7)	< 0.001	0.001	0.667
Pre-LT HBV DNA positivity	2248 (48.0)	168 (34.2)	62 (39.2)	< 0.001	0.030	0.251
HBV DNA $\geqslant 10^5$ copies/mL at LT	1024 (21.9)	40 (8.1)	17 (10.8)	< 0.001	0.001	0.313
Pre-LT antiviral therapy	2604 (55.6)	272 (55.4)	104 (65.8)	0.934	0.011	0.021
Duration of antiviral therapy before LT, mean ± SD (range) (d)	233.4 ± 604.4 (1-7633)	92.8 ± 299.3 (1-3280)	347.1 ± 899.0 (2-7766)	0.804	< 0.001	< 0.001
Number of donors	4684	491	158	-	-	-
Age, mean ± SD (range) (yr)	28.8 ± 6.2 (18-62)	29.1 ± 6.9 (19-61)	29.3 ± 6.3 (20-51)	0.997	0.700	0.737
Male sex	4495 (96.0)	467 (95.1)	147 (93.0)	0.365	0.069	0.315
Deceased donor	4373 (93.4)	424 (86.4)	129 (81.7)	< 0.001	< 0.001	0.147
Living donor	311 (6.6)	67 (13.7)	29 (18.3)	< 0.001	< 0.001	0.147
BMI, mean ± SD (range)	22.4 ± 2.7 (15.5-52.1)	23.2 ± 2.7 (17.6-30.5)	22.6 ± 2.9 (15.8-28.4)	0.069	0.895	0.646
HBsAb positivity	606 (12.9)	68 (13.8)	27 (17.1)	0.568	0.128	0.316
HBcAb positivity	160 (3.4)	21 (4.3)	2 (1.3)	0.323	0.139	0.075

MELD: Model for end-stage liver disease; LT: Liver transplantation; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen; HBV DNA: Hepatitis B virus deoxyribonucleic acid; BMI: Body mass index; HBsAb: Hepatitis B surface antibody; HBcAb: Hepatitis B core antibody.

Table 2 Posttransplant survival of the recipients

	Group A	Group B	Group C	*P* value		
				A <-> B	A <-> C	B <-> C
Recipients (*n*)	4684	491	158	--	--	--
Death during the follow-up (*n*)	939	57	18	--	--	--
Cumulative survival rate						
1-yr	86.2%	94.4%	92.5%			
3-yr	76.9%	86.6%	87.0%	< 0.001	< 0.001	0.137
5-yr	73.7%	82.4%	81.6%			
Duration of follow-up, mean ± SD (range) (mo)	45.8 ± 33.7 (0-141.8)	30.2 ± 17.2 (0.1-77.1)	35.1 ± 20.5 (0.2-84.2)	--	--	--

RESULTS

Baseline characteristics

Table 1 shows the baseline characteristics of the 5333 HBsAg-positive recipients using the prophylactic protocol with one nucleos(t)ide analogue and low-dose im HBIG. No differences existed among the recipients in groups A, B and C with respect to age, gender, pre-LT model for end-stage liver disease and pre-LT Child-Pugh score. However, group A had more recipients with positive HBV DNA and with high viral load (HBV DNA $\geqslant 10^5$ copies/mL) before transplantation than groups B and C. group C had more patients using antiviral therapy and longer duration of antiviral therapy before LT than groups A and B. group B had more patients combined with hepatocellular carcinoma (HCC) than groups A and C. In addition, both groups B and C had more patients with positive HBeAg before LT than group A.

Table 1 also lists the baseline characteristics of the donors. The donors in the three groups had similar characteristics with respect to age, gender, body mass index, percentage of donors with serum positive HBsAb and HBcAb.

Patient survival

As shown in Table 2, 939 recipients died during the follow-up in group A, 57 in group B and 18 in group C. The survival curve for each group is shown in Figure 2A. The 1-, 3- and 5-year survival rates were significantly lower in group A than in groups B (86.2% *vs* 94.4%, 76.9% *vs* 86.6%, 73.7% *vs* 82.4%, respectively, $P < 0.001$) and C (86.2% *vs* 92.5%, 76.9% *vs* 87.0%, and 73.7% *vs* 81.6%, respectively, $P < 0.001$). In addition, the 1-, 3- and 5-year survival rates were 94.4%, 86.6% and 82.4%, respectively, in group B *vs* 92.5%, 87.0% and 81.6%, respectively, in group C ($P = 0.137$).

HBV recurrence

During the follow-up period, 179 patients experienced HBV recurrence in group A, 5 in group B and 3 in group

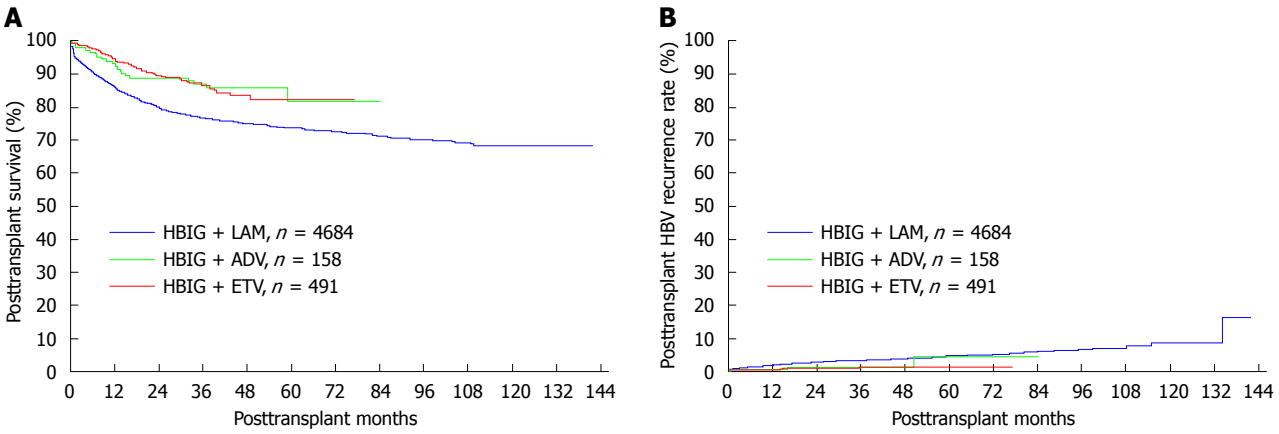

Figure 2 Cumulative posttransplant survival and hepatitis B virus recurrence rates for each group. A: Cumulative posttransplant survival; B: Cumulative posttransplant hepatitis B virus recurrence. HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir dipivoxil.

Table 3 Posttransplant hepatitis B virus recurrence of the recipients

	Group A	Group B	Group C	*P* value		
				A <-> B	A <-> C	B <-> C
Recipients (*n*)	4684	491	158	-	-	-
HBV recurrence during the follow-up (*n*)	179	5	3	-	-	-
Death in patients with HBV recurrence (*n*)	47	2	1	-	-	-
Cumulative HBV recurrence rate						
1-yr	1.7%	0.5%	0.7%			
3-yr	3.5%	1.5%	1.5%	0.023	0.060	0.234
5-yr	4.7%	1.5%	4.4%			

C (Table 3). As shown in Figure 2B, the 1-, 3- and 5-year HBV recurrence rates were significantly higher in group A than in group B (1.7% *vs* 0.5%, 3.5% *vs* 1.5%, 4.7% *vs* 1.5%, respectively, $P = 0.023$). No significant difference existed between groups A and C with respect to the 1-, 3- and 5-year HBV recurrence rates (1.7% *vs* 0.7%, 3.5% *vs* 1.5%, 4.7% *vs* 4.4%, respectively, $P = 0.060$) and between groups B and C with respect to the 1-, 3- and 5-year HBV recurrence rates (0.5% *vs* 0.7%, 1.5% *vs* 1.5%, 1.5% *vs* 4.4%, respectively, $P = 0.234$).

Risk factors for posttransplant HBV recurrence

As shown in Table 4, pre-LT recipient with HCC, serum HBV DNA $\geqslant 10^5$ copies/mL, not using ETV before transplantation, post-LT HBV prophylactic protocol (LAM and HBIG *vs* ETV and HBIG), female donor and donor with negative serum HBsAb were significant risk factors for HBV recurrence by univariate analysis ($P < 0.10$). In multivariate analysis, pre-LT HCC, serum HBV DNA $\geqslant 10^5$ copies/mL and posttransplant HBV prophylactic protocol (LAM and HBIG *vs* ETV and HBIG) were found to be independent predictive factors for posttransplant HBV recurrence ($P < 0.05$) (Table 5).

Cost for the prophylaxis protocols

The cost for group A was approximately $4367 in the first year posttransplantation and $2741 yearly thereafter, and the corresponding figures were $5485 and $3860 for group B, and $4544 and $2918 for group C.

DISCUSSION

One goal of this study was to evaluate the prophylactic effects of low-dose im HBIG and different nucleos(t)ide analogues on posttransplant HBV recurrence in China. Presently, several nucleos(t)ide analogues are available for the treatment of chronic hepatitis B. Of these, ETV, which is a very potent anti-HBV selective guanosine analog, has higher efficacy than LAM or ADV in patients with chronic hepatitis B, therefore resulting in earlier and superior reduction in HBV DNA[9-11]. In addition, ETV is associated with a high genetic barrier to resistance that requires multiple mutations for resistance to emerge. In nucleoside-naive patients, the probability of developing resistance to ETV remained consistently low (< 1.2%) after 96 wk of therapy[12]. In view of the satisfactory outcomes of ETV in the non-transplant setting, ETV and HBIG may be a more effective prophylaxis protocol in transplant recipients than HBIG plus LAM or ADV. However, there are limited data on the use of ETV and HBIG in the transplant setting. To the best of our knowledge, there are three studies on patients receiving ETV and HBIG after LT[13-15]. One representative research was from Ueda *et al*[13] in 2013, in which ETV and HBIG resulted in no HBV recurrence during the median follow-up period of 25.1 mo in 26 patients. However,

Table 4 Univariate Cox proportional hazards analysis for posttransplant hepatitis B virus recurrence

Factor		Hazard ratio	95%CI	*P* value
Age (yr)	18-29 *vs* ≥ 65	2.281	0.589-8.831	0.232
	30-39 *vs* ≥ 65	1.441	0.438-4.736	0.547
	40-49 *vs* ≥ 65	1.910	0.603-6.057	0.272
	50-64 *vs* ≥ 65	1.657	0.522-5.262	0.392
Gender	Male *vs* Female	1.092	0.687-1.737	0.710
Pre-LT MELD score	6-9 *vs* 30-40	1.347	0.789-2.300	0.276
	10-19 *vs* 30-40	1.226	0.767-1.958	0.395
	20-29 *vs* 30-40	1.034	0.613-1.744	0.899
Pre-LT Child-Pugh score	5-6 *vs* 10-15	0.921	0.584-1.452	0.723
	7-9 *vs* 10-15	1.029	0.710-1.492	0.879
Pre-LT with HCC	Yes *vs* No	1.438	1.078-1.919	0.014
Pre-LT HBeAg status	Positive *vs* Negative	1.176	0.956-1.772	0.325
Pre-LT serum HBV DNA level				
HBV DNA	Positive *vs* Negative	1.185	0.805-1.743	0.389
HBV DNA ≥ 10^5 copies/mL	Yes *vs* No	1.395	1.012-1.921	0.042
Pre-LT antiviral therapy				
Using LAM	Yes *vs* No	0.930	0.697-1.241	0.622
Using ETV	Yes *vs* No	0.133	0.019-0.949	0.044
Using ADV	Yes *vs* No	0.328	0.046-2.333	0.265
Post-LT HBV prophylactic protocol	HBIG + LAM *vs* HBIG + ETV	2.949	1.210-7.188	0.017
	HBIG + ADV *vs* HBIG + ETV	1.714	0.410-7.171	0.461
Donor profiles				
Donor source	Living donor *vs* Deceased donor	0.900	0.500-1.621	0.726
Donor gender	Male *vs* Female	0.564	0.298-1.067	0.078
Donor HBsAb positivity	Positive *vs* Negative	0.481	0.267-0.864	0.014
Donor HBcAb positivity	Positive *vs* Negative	1.598	0.786-3.247	0.195

LT: Liver transplantation; MELD: Model for end-stage liver disease; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen; HBV DNA: Hepatitis B virus deoxyribonucleic acid; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir dipivoxil; HBIG: Hepatitis B immunoglobulin; HBsAb: Hepatitis B surface antibody; HBcAb: Hepatitis B core antibody.

Table 5 Multivariate Cox proportional hazards analysis for posttransplant hepatitis B virus recurrence

Factor		Hazard ratio	95%CI	*P* value
Pre-LT with HCC	Yes *vs* No	1.718	1.243-2.375	0.001
Pre-LT serum HBV DNA ≥ 10^5 copies/mL	Yes *vs* No	1.370	0.989-1.897	0.048
Pre-LT using ETV	Yes *vs* No	0.166	0.019-1.484	0.108
Post-LT HBV prophylactic protocol	HBIG + LAM *vs* HBIG + ETV	2.127	0.416-3.055	0.046
Donor profiles				
Donor gender	Male *vs* Female	0.632	0.156-1.144	0.201
Donor HBsAb positivity	Positive *vs* Negative	0.526	0.265-1.045	0.066

LT: Liver transplantation; HCC: Hepatocellular carcinoma; HBV DNA: Hepatitis B virus deoxyribonucleic acid; ETV: Entecavir; HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine; HBsAb: Hepatitis B surface antibody.

these studies were limited due to small size and short follow-up. It is difficult to draw a definite conclusion. Recently, Cholongitas *et al*[16] have published a systematic review about ETV and HBIG after LT. Their findings favor the use of HBIG and an hgbNA such as ETV instead of HBIG combined with LAM for prophylaxis against HBV recurrence after LT. In the nationwide multicenter study, combination prophylaxis with ETV and low-dose im HBIG resulted in 1-, 3- and 5-year HBV recurrence rates of 0.5%, 1.5% and 1.5%, respectively, which were significantly lower than those in group B with LAM and low-dose im HBIG (1-, 3- and 5-year HBV recurrence rates of 1.7%, 3.5% and 4.7%, respectively, $P = 0.023$). Our result definitely reinforces the role of ETV in HBV prophylaxis after LT.

Another goal of this study was to identify the risk factors for posttransplant HBV recurrence. Three factors [pre-LT HCC, serum HBV DNA ≥ 10^5 copies/mL and posttransplant HBV prophylactic protocol (LAM plus HBIG *vs* ETV plus HBIG)] were associated with posttransplant HBV recurrence in our study.

Currently, the role of HCC in posttransplant HBV recurrence remains unclear. Some studies have reported that pre-LT HCC is an important risk factor for HBV recurrence in patients undergoing transplantation[17,18], while others found no association between them[19,20]. In 2008, Faria *et al*[17] found that pre-LT HCC was associated with an increased risk of HBV reinfection after transplantation. Eleven of the 31 patients with HCC at the time of transplantation presented with HBV

recurrence, and 3 of the 68 patients without HCC had HBV recurrence ($P < 0.001$). Recently, Xu *et al*[18] also reported a similar relationship between pre-LT HCC and post-LT HBV recurrence, one potential theoretical explanation for which may be that a large tumor burden on the explants may indicate the presence of extrahepatic, micrometastatic sites, which may serve as a source for HBV replication. The large cohort and long follow-up of this study are enough to evaluate the role of pre-LT HCC in posttransplant HBV recurrence, and our results further verify the close connection between pre-LT HCC and post-LT HBV reinfection. To reduce the impact of this risk factor, potent prophylactic protocols, such as ETV and low-dose im HBIG, may be recommended after LT in patients with pre-LT HCC.

As shown in the literature, positive HBV DNA or high pre-LT viral load has always been an important predictor of HBV reinfection posttransplantation[21-24]. Consistent with previous studies, the present data indicated that a pre-LT viral load greater than 10^5 copies/mL was an independent risk factor for hepatitis B relapse after LT. To reduce the impact of this risk factor, effective antiviral therapy is necessary. However, in practice the duration of antiviral therapy before LT varies among patients because it largely depends on the predictability of transplant timing. Therefore, the goal of reducing the HBV DNA level sufficiently prior to LT may not be achieved in every recipient.

As mentioned before, combination therapy with ETV and low-dose im HBIG has been proven to be a potent prophylactic protocol. In contrast, the regimen with LAM and low-dose im HBIG resulted in a higher rate of HBV recurrence. The relative weak prophylactic efficacy of LAM and HBIG compared with that of ETV and HBIG was also proven by both univariate and multivariate analyses. Therefore, ETV and HBIG may be considered an efficient therapy for the prevention of HBV recurrence after transplantation.

In addition, the predictive value of the pre-LT HBeAg status on HBV relapse posttransplantation remains controversial. Steinmüller *et al*[25] found that post-LT HBV recurrence rate was associated significantly with the preoperative HBeAg status. Patients in the positive HBeAg group showed a significantly higher recurrence rate than HBeAg-negative patients. In contrast, other studies reported negative results[24,26]. In this study, no significant difference was observed between the recipients with serum positive HBeAg and those with negative HBeAg (Table 4). It appears that preoperative HBeAg status is less valuable than the HBV DNA in predicting HBV recurrence.

The present study has several limitations, mainly based on its retrospective nature. We could not evaluate the prophylactic efficacy of the regimen with telbivudine and low-dose im HBIG because of the small size sample (45 cases), which could not reach a statistical significance. We also could not acquire detailed data on the post-transplant resistance of oral antiviral drugs. However, the large size (5333 cases) and long follow-up (mean, 42.1 ± 30.3 mo) of this current study has enabled accurate evaluation of prophylactic efficacy of different regimens and potential predictors of posttransplantation HBV recurrence.

In conclusion, this nationwide multicenter study demonstrated that low-dose im HBIG and one nucleos(t)ide analogue provides an effective prophylaxis against recurrent HBV infection posttransplantation at approximately 5% of the cost of conventional high-dose iv HBIG regimens. Among them, low-dose im HBIG combined with ETV has better prophylactic efficacy than the combination therapy with low-dose im HBIG and LAM. Thus, we suggest that ETV and low-dose HBIG should be considered an efficient therapy in our country instead of LAM and low-dose HBIG. In addition, three factors [pre-LT HCC, serum positive HBV DNA $\geqslant 10^5$ copies/mL and posttransplant HBV prophylactic protocol (LAM and HBIG *vs* ETV and HBIG)] were associated with posttransplant HBV recurrence in our study.

ACKNOWLEDGMENTS

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COMMENTS

Background

Hepatitis B virus (HBV)-related liver diseases account for approximately 78% of all adult liver transplant recipients. However, the main issue in hepatitis B surface antigen (HBsAg)-positive recipients is the risk of HBV recurrence posttransplantation, which may lead to rapid disease progression or even death. With the introduce of new nucleos(t)ide analogues, such as adefovir dipivoxil, telbivudine and entecavir (ETV), some centers also chose the protocol with another nucleos(t)ide analogue and intramuscular (im) hepatitis B immunoglobulin (HBIG) to prevent HBV reinfection after liver transplantation (LT).

Research frontiers

Currently, little is known about which protocol has the optimal prophylactic effects against HBV recurrence. Authors use the data from China Liver Transplant Registry database to evaluate the long-term prophylactic efficacy of HBIG plus different nucleos(t)ide analogue and find the risk factors for HBV recurrence. Among them, low-dose intramuscular HBIG combined with ETV has better prophylactic effect than the combination therapy with low-dose intramuscular HBIG and lamivudine (LAM).

Innovations and breakthroughs

The results suggest that low-dose intramuscular HBIG combined with ETV has better prophylactic effect than the combination therapy with low-dose intramuscular HBIG and LAM.

Applications

Authors suggest that ETV plus low-dose HBIG should be considered an efficient therapy in their country instead of LAM and low-dose HBIG.

Terminology

LT is the replacement of a diseased liver with part or all of a healthy liver from another person. In patients with end-stage HBV-related liver diseases, LT offers a life-saving treatment. However, the main issue in HBsAg-positive recipients is the risk of HBV recurrence posttransplantation, which may lead to rapid disease.

Peer review

The paper reports on the results of the China Liver Transplant Registry on HBV prophylaxis in patients receiving liver transplantation. They conclude that a

lower dose of HBIG plus adefovir or entecavir or lamivudine results in excellent treatment response, especially the combination HBIG/entecavir. The paper is well written and of highly clinical implications.

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ORIGINAL ARTICLE

Clinical Trials Study

Short turn radius colonoscope in an anatomical model: Retroflexed withdrawal and detection of hidden polyps

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Author contributions: McGill SK, Kothari S, Friedland S and Banerjee S conceived and designed this study, drafted the article, performed analysis and approved the final version; Chen A and Park WG contributed to analysis, drafting of article and approved the final version.

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Abstract

AIM: To evaluate the new RetroView™ colonoscope and compare its ability to detect simulated polyps "hidden" behind colonic folds with that of a conventional colonoscope, utilizing anatomic colon models.

METHODS: Three anatomic colon models were prepared, with twelve simulated polyps "hidden" behind haustral folds and five placed in easily viewed locations in each model. Five blinded endoscopists examined two colon models in random order with the conventional or RetroView™ colonoscope, utilizing standard withdrawal technique. The third colon model was then examined with the RetroView™ colonoscope withdrawn initially in retroflexion and then in standard withdrawal. Polyp detection rates during standard and retroflexed withdrawal of the conventional and RetroView™ colonoscopes were determined. Polyp detection rates for combined standard and retroflexed withdrawal (combination withdrawal) with the RetroView™ colonoscope were also determined.

RESULTS: For hidden polyps, retroflexed withdrawal using the RetroView™ colonoscope detected more polyps than the conventional colonoscope in standard withdrawal (85% *vs* 12%, $P = 0.0001$). For hidden polyps, combination withdrawal with the RetroView™ colonoscope detected more polyps than the conventional colonoscope in standard withdrawal (93% *vs* 12%, $P \leqslant 0.0001$). The RetroView™ colonoscope in "combination withdrawal" was superior to other methods in detecting all (hidden + easily visible) polyps, with successful detection of 80 of 85 polyps (94%) compared to 28 (32%) polyps detected by the conventional colonoscope in standard withdrawal ($P < 0.0001$) and 67 (79%) polyps detected by the RetroView™ colonoscope in retroflexed withdrawal alone ($P < 0.01$). Continuous withdrawal of the colonoscope through the colon model while retroflexed was achieved by all endoscopists. In a post-test survey, four out of five colonoscopists reported that manipulation of the colonoscope was easy or very easy.

CONCLUSION: In simulated testing, the RetroView™ colonoscope increased detection of hidden polyps. Combining standard withdrawal with retroflexed withdrawal may become the new paradigm for "complete screening colonoscopy".

Key words: Colonoscopy; Adenoma detection; Polyp detection; Colonoscope retroflexion; Colon cancer

Core tip: Polyps located on the proximal side of colon folds can be challenging to detect. The new RetroView™ colonoscope has a short turning radius that allows a retroflexed view of the colon during withdrawal. In this bench colon model study, the RetroView™ colonoscope detected more proximally-located, "hidden" polyps during retroflexed withdrawal, than a conventional colonoscope withdrawn in standard fashion. The highest polyp detection rate was achieved when the RetroView™ colonoscope was withdrawn in retroflexion followed by standard withdrawal. This combination of standard and retroflexed withdrawal holds promise for optimizing polyp detection in patients undergoing screening colonoscopy.

McGill SK, Kothari S, Friedland S, Chen A, Park WG, Banerjee S. Short turn radius colonoscope in an anatomical model: Retroflexed withdrawal and detection of hidden polyps. *World J Gastroenterol* 2015; 21(2): 593-599 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/593.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.593

INTRODUCTION

Colorectal cancer is the second most common cause of cancer related mortality in the United States with over 50000 deaths reported annually[1]. Colonoscopy is widely considered the optimal screening modality for colorectal cancer[2], and has been widely adopted for this purpose since Medicare coverage for screening colonoscopy was initiated in 2001. However, interval colorectal cancers following colonoscopy do occur, indicating that colonoscopy offers incomplete protection, particularly in the right colon[3-7]. Interval cancers may arise as a consequence of differential tumor biology, incompletely resected polyps or polyps that are entirely missed at colonoscopy. That polyps are missed at colonoscopy has long been evident. Three tandem colonoscopy studies performed over the last two decades have indicated that colonoscopy is associated with a significant polyp miss rate, with around 6%-27% of adenomas missed at colonoscopy, with the higher miss rates noted for smaller polyps[8-10].

Multiple factors may contribute to polyps being missed at colonoscopy, including suboptimal bowel preparation, inadequate colonic distension, unrecognized flat polyps, and inadequate endoscopy technique, particularly rapid colonoscope withdrawal. A significant additional factor is that polyps located on the proximal aspect of colonic haustral folds, flexures and valves may be missed due to the difficulties in visualizing these areas using conventional colonoscopes and standard withdrawal techniques[11,12]. When used in conjunction with standard withdrawal, retroflexion of a conventional colonoscope in the right colon with withdrawal in retroflexion up to the hepatic flexure (allowing visualization of the proximal aspect of colonic folds), was shown to detect additional polyps in 5.8% of patients undergoing screening or surveillance colonoscopy[13].

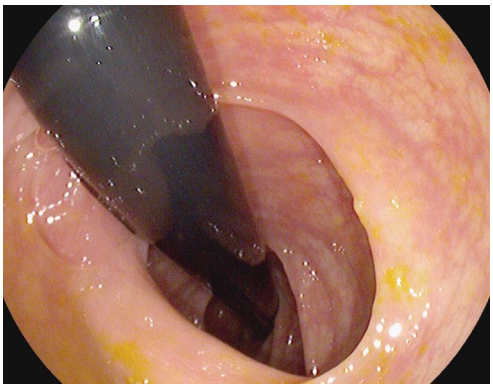

Figure 1 RetroView™ colonoscope being withdrawn in retroflexion through a patient's descending colon.

Colonoscope technology has evolved mainly on the optical front, with incorporation of high definition imaging, wide angled lenses and electronic chromoendoscopy such as narrow band imaging (NBI, Olympus America, Center Valley, PA), i-SCAN™ (PENTAX of America, Montvale, NJ) and Fuji Intelligent Chromo-Endoscopy (FICE™, Fujinon Endoscopy, Wayne, NJ). However, there has been no significant evolution of the mechanical ability of colonoscopes over the last two decades and visualizing the proximal aspects of folds, flexures and valves remains a challenge. Although use of a disposable retrograde viewing device advanced *via* the accessory channel of a standard colonoscope was shown to increase adenoma detection[14-17], this device has not been widely adopted due to technical, cost and payer issues.

The PENTAX EC-3490TLi RetroView™ is a new slim colonoscope with a short turn radius (STR) at the colonoscope's tip, allowing easy retroflexion in the right colon or transverse colon (Video 1). In addition, it has a relatively narrow retroflexed profile, which potentially allows complete withdrawal in full retroflexion from the cecum to the rectum in many patients (Figure 1). This may allow for detection of polyps hidden behind flexures, folds and valves, which may not be seen during a standard "forward viewing" withdrawal.

Our objective was to compare the ability of the PENTAX RetroView™ colonoscope with that of a conventional slim colonoscope in detecting simulated hidden polyps in an anatomic colonic model, particularly those situated behind flexures and folds. In this study, the polyp detection rate of the RetroView™ colonoscope on retroflexed withdrawal, standard withdrawal and "combination" withdrawal (retroflexed + standard withdrawal) was compared to that of the conventional colonoscope

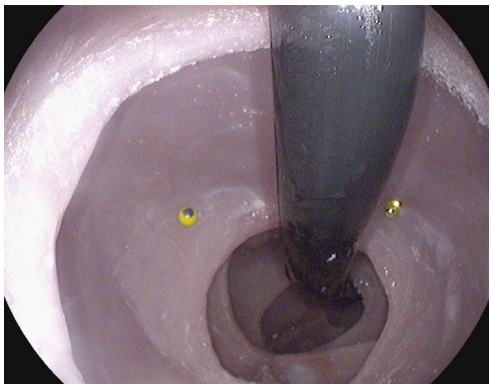

Figure 2 Colonic model with two simulated polyps on the proximal aspect of a fold, visualized using the RetroView™ colonoscope in retroflexed withdrawal.

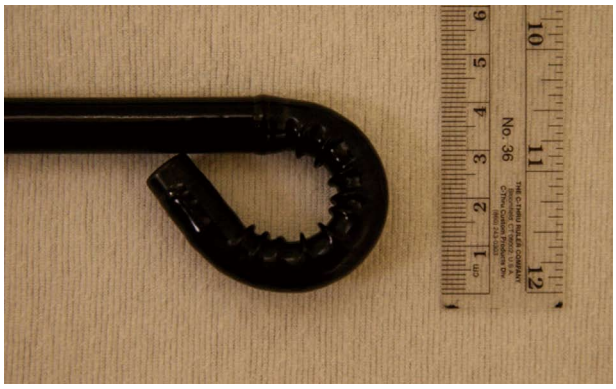

Figure 3 Retroflexed RetroView™ colonoscope.

on standard withdrawal.

MATERIALS AND METHODS

Three identical, realistic colon models constructed of silicone (DeLegge Medical, Mt. Pleasant, SC) incorporating anatomically correct haustral folds and flexures, with a colonic length of 127 cm were utilized for this study (Figure 2).

Simulated polyps comprised of beads of various colors measuring 4 mm wide and 3 mm high, held in place by metal pins. Seventeen polyps were placed in each of the three colon models; 12 (79%) were positioned on the proximal aspects of folds or flexures and 5 (21%) were positioned in "obvious" locations, where they would be expected to be seen on standard withdrawal. A "perfect score" would occur if the five endoscopists identified all 17 polyps in the colon model, for a total of 85 polyps. The location of bead placement, the order of colors and number of beads of each color were different for each model, to avoid a learning effect as the endoscopists evaluated the models sequentially.

Two colonoscopes were used in this study: a conventional slim colonoscope (EC 3490K, PENTAX, Montvale, NJ) and the RetroView™ colonoscope (EC-3490TLi, PENTAX, Montvale, NJ). The RetroView™ colonoscope is visually identical to a conventional slim colonoscope. It is however unique in that it has a short turning radius and in the fully retroflexed position, the maximal width of the bending section (*i.e.*, distance including main scope shaft and retroflexed shaft) is only 40 mm (Figure 3).

Five endoscopists with varying levels of experience participated in the study. A technical team prepared each model by advancing the selected colonoscopes to the cecum. The conventional colonoscope was advanced to the cecum in one model and the RetroView™ colonoscope was advanced to the cecum in two of the models. Each endoscopist first examined two models sequentially in random order with either the conventional colonoscope or the RetroView™ colonoscope utilizing standard withdrawal technique. They were blinded as to the type of colonoscope being used for these two colon models. They were asked to describe the unique color of each simulated polyp seen. Endoscopists were requested to limit their withdrawal time to 6 min.

The third model was then examined with the RetroView™ colonoscope initially in complete retroflexion by the endoscopist, and the number of simulated polyps detected was noted. This was followed by a standard withdrawal using the same colonoscope. Blinding to the colonoscope used for the third model was not possible, given the RetroView™ colonoscope's unique ability to retroflex easily and need to be withdrawn in retroflexion for this study. The total number of polyps found by standard withdrawal and retroflexed withdrawal using the RetroView™ colonoscope were summed to determine the polyp detection rate with a "combination withdrawal". The RetroView™ colonoscope's ability to be withdrawn in complete retroflexion by each endoscopist all the way from the cecum to rectum in these models was noted.

Following the examination, endoscopists filled out a "post-test" questionnaire that asked about the overall ease of use of the RetroView™ colonoscope, the ease of manipulating and withdrawing the colonoscope in retroflexion and the ease of re-orientating to retroflexed views during colonoscope withdrawal. Optional responses to these questions were: very easy, easy, somewhat difficult, difficult or very difficult. At the time of this study, the RetroView™ colonoscope was not commercially available and the endoscopists were also were asked whether they would consider performing additional routine retroflexed withdrawal at colonoscopy when the colonoscope became commercially available.

Statistical comparisons were performed with use of the Cochran-Mantel-Haenszel test, stratifying the data by endoscopist.

RESULTS

The results were summed among the five endoscopists, and are shown in Table 1.

Hidden polyps

The RetroView™ colonoscope on retroflexed withdrawal detected more hidden polyps located on the proximal

Table 1 Cumulative polyp detection rates by colonoscope and withdrawal method *n* (%)

	A Conventional colonoscope, Standard Withdrawal	B RetroView™ colonoscope, Standard Withdrawal	C RetroView™ colonoscope, Retroflexed Withdrawal	D RetroView™ colonoscope, Combination Withdrawal (Standard + Retroflexed)	*P* values
Hidden polyps (n = 60)	7 (12)	11 (18)	51 (85)	56 (93)	A *vs* B, P = 0.5 A *vs* C, P < 0.0001 A *vs* D, P < 0.0001
Obvious polyps (n =25)	21 (84)	19 (76)	16 (64)	24 (96)	A *vs* B, P = 0.4 A *vs* C, P = 0.7 A *vs* D, P < 0.0001 C *vs* D, P = 0.01
All polyps (n =85)	28 (32)	30 (35)	67 (79)	80 (94)	A *vs* C, P < 0.001 A *vs* D, P < 0.0001 C *vs* D, P < 0.01

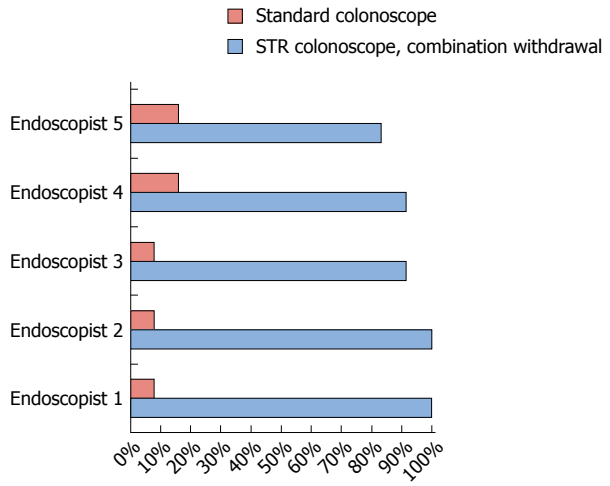

Figure 4 Detection rates for hidden polyps by endoscopist, comparing the conventional colonoscope in standard withdrawal and the RetroView™ colonoscope in combination withdrawal.

aspects of folds compared to the conventional colonoscope on standard withdrawal, finding 51 of 60 (85%) such polyps, compared to just seven (12%) detected by the conventional colonoscope on standard withdrawal (P < 0.0001). Combination withdrawal yielded the highest detection rate, finding 56 (93%) of hidden polyps (P < 0.0001 *vs* conventional colonoscope). The RetroView™ colonoscope and conventional colonoscope detected similar numbers of polyps on standard withdrawal, 11 (18%) *vs* 7 (12%), (P = 0.5).

Endoscopists' individual detection rates for hidden polyps ranged from 8%-17% using the conventional colonoscope, from 83%-92% using the RetroView™ colonoscope in retroflexion, and from 83%-100% for the RetroView™ colonoscope using combination withdrawal (Figure 4).

Obvious polyps

The RetroView™ colonoscope had a similar detection rate for obvious polyps in standard withdrawal as the conventional colonoscope in standard withdrawal, finding 19 polyps of the total 25 (76%) *vs* 21 polyps (84%) detected by the conventional colonoscope (P = 0.5). Combination withdrawal with the RetroView™ colonoscope found similar numbers of obvious polyps as the conventional colonoscope, finding 24 (96%) of such polyps (P = 0.36). The RetroView™ colonoscope had a lower detection rate for obvious polyps when retroflexed than when used in combination withdrawal-16 (64%) *vs* 24 (96%) (P = 0.01).

All polyps

The RetroView™ colonoscope in "combination withdrawal" was superior to other methods in detecting all (hidden + easily visible) polyps, with successful detection of 80 of 85 polyps (94%) compared to 28 (32%) polyps detected by the conventional colonoscope in standard withdrawal (P < 0.0001) and 67 (79%) polyps detected by the RetroView™ colonoscope in retroflexed withdrawal alone (P < 0.01).

Ability to retroflex and withdrawal time: Complete retroflexed withdrawal with the RetroView™ colonoscope in the realistic anatomical colon model was achieved by all endosocopists. Average withdrawal time with the conventional colonoscope was 4 min 30 s. For the RetroView™ colonoscope, average withdrawal time was 4 min 24 s for standard withdrawal, 3 min 50 s for retroflexed withdrawal and 8 min and 8 s for combination withdrawal.

There was no individual polyp that was never detected by all of the examiners, indicating that misses were not the result of inadequate visualization.

Post-test questionnaire: On the post-test questionnaire, all participants indicated that overall, the RetroView™ colonoscope was either easy or very easy to use. Four endoscopists described manipulation of the RetroView™ colonoscope during retroflexed withdrawal as easy or very easy, while one described this as difficult. All endoscopists indicated that they would perform additional routine retroflexed withdrawal at colonoscopy when the RetroView™ colonoscope became commercially available.

DISCUSSION

Our study indicates that the RetroView™ colonoscope, in simulated testing using retroflexed or combination withdrawal, significantly improves the detection of "hidden" polyps located on the proximal aspect of colonic folds, compared to standard withdrawal using a conventional colonoscope. The highest detection rates for all polyps, both those that were hidden and placed in obvious locations, were achieved with combination withdrawal of the RetroView™ colonoscope.

Missed adenomas may lead to interval colon cancers and diminish the efficacy of colonoscopy[7], and prior studies suggest that the proximal aspects of colonic folds, flexures and valves are a common site for missed polyps[11,12]. An early study that appraised polyps that were identified by barium enema but missed at endoscopy demonstrated that missed lesions had a tendency to be located on the proximal aspects of haustral folds and valves[11]. More recently, Pickhardt *et al*[12] also demonstrated that 71.4% of non-rectal adenomas ⩾ 6 mm in size missed at colonoscopy but detected at CT colonography, were located on the proximal side of colonic folds.

Thus visualization of the proximal aspects of colonic folds is desirable. Indeed, retroflexion of standard colonoscopes in the right colon was shown to increase the adenoma yield in a large study[13]. Potentially, this additional yield of "missed polyps" might be higher if the entire colon could be viewed in retroflexed withdrawal, in addition to the standard forward viewing withdrawal. However, with standard colonoscopes, retroflexed withdrawal is typically only possible in the right colon, due to their larger turn radius and width of the bending section when retroflexed. There has been no significant evolution in the mechanical ability of colonoscopes over the last two decades to address this issue. As a consequence, other techniques and technologies have emerged to address this unmet need, with variable results.

Colonoscopes incorporating a 170 degree wide angled lens rather than the standard 140 degree lens were introduced with the hope of improving polyp detection. However, clinical studies indicate that this colonoscope did not improve adenoma detection[18,19] or miss rates[20] in trials but only increased the discovery of small hyperplastic polyps[18]. Translucent caps have been fitted to the tip of colonoscopes to assist with depressing haustral folds to potentially improve colonic visualization and polyp detection. Again, studies evaluating adenoma detection rates with cap fitted colonoscopy have yielded mixed results[21-26], and it is unclear if this technique is beneficial. Similarly studies have indicated that high definition colonoscopes did not improve adenoma detection rates compared to older standard definition colonoscopes[27,28]. Finally, several studies comparing NBI and FICE with white light colonoscopy did not show any increase in adenoma detection rates[29-34].

The largest increase in the detection of additional polyps, over those detected by standard colonoscopy, have been reported with the Third Eye Retroscope (Avantis Medical Systems, Sunnyvale, CA), an auxiliary viewing device which allows retrograde views behind colonic folds and flexures. The device is advanced through the accessory channel of a standard colonoscope and when used in conjunction with the colonoscope, allows the endoscopist simultaneous forward and retrograde facing views of the colon[14,16,17].

The Third Eye Retroscope increased adenoma detection rates by 11%-25%[14-17], but has failed to be widely adopted due to several cost and technical issues. Utilizing this technology requires the purchase of a separate processor and of a new disposable device for each colonoscopy procedure, the cost of which is not reimbursed by most payers. The device occupies the working channel of the colonoscope which limits the ability to suction. This necessitates washing and suctioning of the colon during the colonoscope insertion phase, in cases of suboptimal bowel preparation. If a polyp is seen on the proximal aspect of a colonic fold, the viewing device has to be removed in order that a polypectomy device may be advanced. This may result in loss of visualization of the hidden polyp. The optics of the device are standard rather than high definition and are further impaired by the glare consequent upon the two light sources and lens systems, of the device and colonoscope, that face each other. Finally, the endoscopist has to get used to visualizing and processing two simultaneous video streams from the colonoscope and from the retroscope device.

In contrast, the RetroView™ colonoscope that we tested offers many advantages. It offers the ability to provide high definition views of the proximal aspects of colonic folds, flexures and valves with no additional equipment or device costs. The image is high definition and the colonoscope also incorporates zoom and electronic chromoendoscopy (i-SCAN) abilities. The suction/work channel of the colonoscope is unimpaired and available for use and detected polyps remain in view while polypectomy devices are advanced. Polypectomy can be performed with the colonoscope in retroflexion, without losing views of the polyp[35]. The main "cost" of using the colonoscope in both standard and retroflexed withdrawal, is the additional time necessary for colonoscope reinsertion and retroflexed withdrawal, which will result in a longer overall procedure time.

The results showed that the RetroView™ colonoscope in retroflexion detected fewer obvious polyps than combination withdrawal. As a small portion of the colon is obscured by the shaft of the colonoscope in retroflexion, full visualization of the colonic mucosa requires continuous back and forth torque during withdrawal. Not all of the study endoscopists performed this maneuver. The reduced detection rate of obvious polyps in retroflexion may have reflected this fact.

There are limitations to the current study. The anatomic colon model is stiff and its folds may not be "ironed out" with the colonoscope like the folds of a human colon, possibly making the detection of hidden polyps more difficult than in real life situations.

In conclusion, the RetroView™ colonoscope allowed

withdrawal in complete retroflexion over the entire length of the anatomic colon model. It increased detection of polyps that were hidden behind folds and flexures. Combining standard withdrawal with retroflexed withdrawal promises to increase polyp detection rates and may become the new paradigm for "complete screening colonoscopy". Studies are currently underway at our institution evaluating the RetroView™ colonoscope in human subjects.

COMMENTS

Background

Colonoscopy cancer is widely considered the optimal screening modality for colorectal cancer. However, polyps may be missed at colonoscopy, and these missed polyps can, in turn, potentially evolve into cancer. Polyps located on the proximal aspect of colonic folds, flexures and valves may be particularly difficult to visualize with conventional colonoscopes and using standard colonoscope withdrawal techniques.

Research frontiers

Colonoscope and device innovations have emerged to address the issue of missed polyps. The Retroview™ colonoscope has a short turn radius that allows for easier retroflexion, to better visualize polyps located behind folds and flexures. This complements other advances in colonoscope and device technologies that improve polyp detection.

Innovations and breakthroughs

Compared to conventional colonoscopes, the tip of the RetroView™ colonoscope has a short turning radius, allowing for a narrow retroflexed profile and the potential to withdraw the colonoscope in retroflexion across most or all of the colon, in addition to a standard forward looking withdrawal. This combination of standard forward looking and retroflexed withdrawals allows visualization of both sides of colonic folds and flexures and may therefore improve polyp detection at colonoscopy. In the first known study to evaluate this new colonoscope, the authors describe its performance in a colon model.

Applications

The study suggests that the RetroView™ colonoscope can enhance polyp detection and thus may potentially improve colon cancer prevention by colonoscopy.

Terminology

A "retroflexed view" is one in which the colonoscope tip is turned 180 degrees in order to look backwards. This allows excellent visualization of the proximal aspect of colonic folds.

Peer review

This study is a first step in evaluating the RetroView™ colonoscope and its potential to improve colon polyp detection in humans. The authors evaluated the new colonoscope in finding "hidden" simulated colon polyps in a colon model, and found its performance to be excellent. Use of the RetroView™ colonoscope, utilizing a combination of standard forward looking and retroflexed withdrawals, holds the potential to improve polyp detection in patients undergoing screening colonoscopy.

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ORIGINAL ARTICLE

Clinical Trials Study

Intervention to increase physical activity in irritable bowel syndrome shows long-term positive effects

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Abstract

AIM: To assess the long-term effects of physical activity on irritable bowel syndrome (IBS) symptoms and on quality of life, fatigue, depression and anxiety.

METHODS: Seventy-six patients from a previous randomized controlled interventional study on increased physical activity in IBS were asked to participate in this long-term follow-up study. The included patients attended one visit in which they filled out questionnaires and they underwent a submaximal cycle ergometer test. The primary end point was the change in the IBS Severity Scoring System (IBS-SSS) at baseline, *i.e.*, before the intervention and at follow-up. The secondary endpoints were changes in quality of life, fatigue, depression and anxiety.

RESULTS: A total of 39 [32 women, median age 45 (28-61) years] patients were included in this follow-up. Median follow-up time was 5.2 (range: 3.8-6.2) years. The IBS symptoms were improved compared with baseline [IBS-SSS: 276 (169-360) *vs* 218 (82-328), $P = 0.001$]. This was also true for the majority of the dimensions of psychological symptoms such as disease specific quality of life, fatigue, depression and anxiety. The reported time of physical activity during the week before the visit had increased from 3.2 (0.0-10.0) h at baseline to 5.2 (0.0-15.0) h at follow-up, $P = 0.019$. The most common activities reported were walking, aerobics and cycling. There was no significant difference in the oxygen uptake 31.8 (19.7-45.8) mL per min per kg at baseline *vs* 34.6 (19.0-54.6) mL/min per kg at follow-up.

CONCLUSION: An intervention to increase physical activity has positive long-term effects on IBS symptoms and psychological symptoms.

Key words: Gastrointestinal diseases; Irritable bowel

syndrome; Exercise; Follow-up; Physical activity

Core tip: Increased physical activity for 12 wk has been shown to improve irritable bowel syndrome (IBS) symptoms. This follow-up study found that the patients included in an intervention to increase physical activity show improvements in IBS symptoms, as well as different aspects of the disease specific quality of life, fatigue, depression and anxiety on the long term. The study supports the evidence for the positive effects of physical activity in IBS and defends physical activity as a treatment option for IBS.

Johannesson E, Ringström G, Abrahamsson H, Sadik R. Intervention to increase physical activity in irritable bowel syndrome shows long-term positive effects. *World J Gastroenterol* 2015; 21(2): 600-608 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/600.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.600

INTRODUCTION

In a recent randomized controlled study moderately increased physical activity for 12 wk was tested as an intervention in irritable bowel syndrome (IBS)[1]. Improvements in IBS symptoms as well as in some aspects of the disease specific quality of life were observed in the intervention group. However, there were no significant improvements in fatigue, depression or anxiety. These data demonstrated that moderately increased physical activity for 12 wk improves IBS symptoms without a general improvement of other associated symptoms.

Physical activity has been found to improve a wide range of diseases like fibromyalgia, depression, hypertension and diabetes mellitus. In general these conditions were improved in the short-term but the level of physical activity usually decreases after 12 mo and the benefits of the intervention may be difficult to maintain[2-4]. In some patient groups it is difficult to motivate the patients to change their life style[2,4].

The aim of this study was to assess the long-term effects of the previous intervention to increase physical activity in IBS patients. The primary end point was to assess the change in the IBS Severity Scoring System (IBS-SSS). The secondary endpoints were changes in quality of life, fatigue, depression and anxiety. Moreover, assessments of the level of physical activity and oxygen uptake at follow-up were included.

MATERIALS AND METHODS

Patients

In the previous study the patients were randomized to a physical activity group or to a control group. The physical activity group were instructed by a physiotherapist to increase their physical activity and the control group were instructed to maintain their lifestyle[1]. Patients in the physical activity group were given individual advice depending on their previous level of physical activity and experience of exercise. The activities suggested could be any activity depending on individual factors, such as time, opportunities, or costs. After 12 wk the control group was also instructed by a physiotherapist to increase their physical activity as in the intervention group and they followed the same protocol as the intervention group during the next 12 wk. Both the intervention group and the control group in the previous study were therefore instructed to increase their physical activity before the end of the previous study. Thus all patients completing the previous study had an intervention and were evaluated at the end of the intervention.

In the present study all eligible patients from the previous study were invited to participate in this long-term follow-up. The inclusion criterion was that the patients had baseline data. Baseline data was the data from the first visit of each patient in the previous study before the start of the 12 wk intervention or control period. The exclusion criteria were pregnancy, organic GI disorders or other organic disease hindering physical activity. Seventy-six patients had baseline data and were invited to participate as shown in the flow chart (Figure 1).

The patients were invited by mail in July 2011 and by telephone in August and September 2011. The visits were conducted at the Sahlgrenska University Hospital in Gothenburg, Sweden, during September and October 2011. The subjects attended one visit in which they filled out questionnaires, underwent a submaximal cycle ergometer test and their body weight was registered. The week before the visit the patients registered their bowel movements and physical activities in a paper diary. They were also asked about other IBS related treatments in the years between the baseline visit and the follow-up. The results were compared with the baseline data and the data from the end of the intervention, *i.e.*, at 12 wk. All the subjects gave informed consent, and the study was approved by the Regional Ethical Review Board of the University of Gothenburg, Dnr 091-05.

Questionnaires

IBS-SSS: The IBS-SSS[5] consists of visual analogue scales and is divided into two subscales, an overall IBS score and an extra colonic score. The IBS score contains questions regarding pain severity, pain frequency, abdominal bloating, bowel habit dissatisfaction, and life interference. The extra colonic score contains questions regarding vomiting, gas, belching, satiety, headache, fatigue, musculoskeletal pain, heartburn, dysuria and urgency. Each subscale ranges from 0 to 500, with higher scores meaning more severe symptoms. A reduction of 50 in the IBS-score is considered to be adequate to detect a clinical improvement[5].

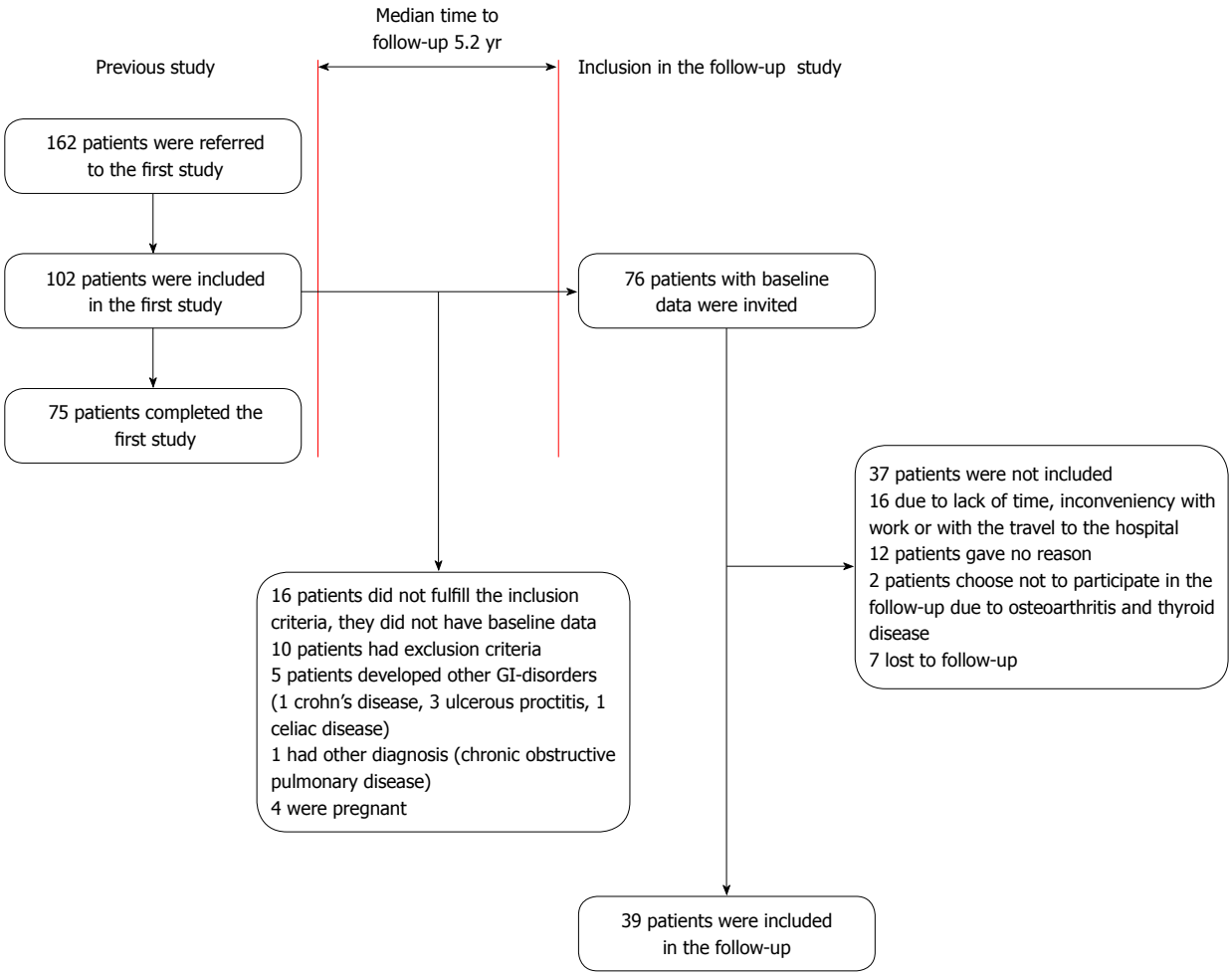

Figure 1 Inclusion of patients in the previous study and the follow-up study. GI: Gastrointestinal.

Bristol stool form scale: Bristol stool form scale was used to record bowel movements during the week before the visits to the laboratory. The patients recorded all bowel movements and its consistency according to the Bristol stool form scale[6]. The scale ranges from 1 to 7, where type 1 and 2 is hard and lumpy stools and type 6 and 7 is loose and watery stools.

The hospital anxiety and depression scale: The hospital anxiety and depression scale (HADS) is a reliable instrument that was developed for medical outpatients[7] and consists of 14 items, each using a 4-graded Likert scale (0-3). The scale is divided into two subscales, anxiety and depression. Each subscale ranges from 0 to 21, where high score indicates more severe symptoms. Cut-off scores can be used to identify cases of clinically significant mood disorder for both subscales. A score of up to 7 indicates no mood disorder while scores of 8-10 show a borderline mood disorder and a score above 10 shows a case of mood disorder. In our analysis scores of 8 and above are considered to indicate clinical symptoms of depression and anxiety.

IBS-quality of life: The IBS-quality of life (IBS-QOL) is a disease specific instrument measuring health-related quality of life (HRQOL). It consists of 30 items which measures nine QOL dimensions: emotional functioning, mental health, sleep, energy, physical functioning, diet, social role, physical role and sexual relations. For each subscale the scores are transformed to range from 0 to 100; 100 representing the best possible disease specific quality of life[8,9].

Short form-36: Short form-36 (SF-36) was used to assess the general HRQOL. SF-36 includes 36 items which are divided into eight subscales: physical functioning, physical role, bodily pain, general health perceptions, vitality, social functioning, emotional role and mental health. For each subscale the raw scores are transformed into a scale from 0 to 100, with 100 representing the best possible HRQOL[10].

Fatigue impact scale: This scale was initially developed for patients with chronic fatigue syndrome[11] and has previously been used in studies in IBS patients[12,13]. The scale consists of 40 questions divided into three subscales: physical functioning (10 items), cognitive functioning (10 items) and psychosocial functioning (20 items). The subjects are asked to rate to which extent fatigue has caused problems for them during the previous

Table 1 Medications used by the patients only at follow-up

Drug	Patients (*n*)
Antacids	1
Neuroleptic drug	1
Drug against neuropathic pain	1
Anti epileptic drug	1
Acetaminophen	3
Antidepressant	3
B_{12} vitamine	3
Post climacteric hormone	1
Anticonceptive	2
NSAIDs	2
Sedative	1

NSAIDs: Non-steroidal anti-inflammatory drugs.

month. Each item consists of a statement and the subject should rate 0 to 4 where 0 means "no problem" and 4 means "extreme problem".

Physical activity and weight

Weight was measured to the nearest 0.1 kg and oxygen uptake was calculated from a submaximal cycle (Monark Ergomedic 839) ergometer test according to Astrand *et al*[14,15]. A training diary was used to register physical activity for one week before the visit. The patients reported the type of physical activity and the duration and intensity of the activity. The patients were instructed to record the time they started the activity and the time they finished. The intensity was rated on Borgs rating of perceived exertion scale[16] which starts at 6, no exertion at all and goes to 20, maximal exertion.

Statistical analysis

The primary endpoint was changes in IBS-SSS, at baseline compared with follow-up. The secondary endpoints were to assess changes in other questionnaires assessing QOL, anxiety, depression, fatigue and bowel movements and changes in oxygen uptake, weight, and time of physical activity reported in the training diary.

As an exploratory endpoint, changes in the above-mentioned parameters in the period between the end of the intervention and the follow-up visit were assessed.

The results are presented as median, percentile 10 and 90. Paired *t* test was only used for oxygen uptake and body weight. The results from the questionnaires were considered as ordinal data, and analyses were performed using Wilcoxon's signed rank test. Significance was accepted at the 5% level ($P < 0.05$). For statistical analysis we used SPSS version 20 (SPSS, IBM; Corporation, NY).

RESULTS

Patients

A total of 39 [32 women, median age 45 (28-61) years] patients were included in the follow-up as shown in the flow chart (Figure 1). According to the Rome Ⅱ criteria[17] 13 patients were classified as diarrhea-predominant IBS, 11 were classified as constipation-predominant IBS and 15 as alternating IBS. Patients who were not included in the follow-up are reported in Figure 1. In total 33 patients attended the follow-up visit while six patients participated by completing the questionnaires due to inconveniency for them to come to the visit.

Thirty-seven of the included patients participated in the previous intervention either as a part of the intervention group (19 patients) or as a control group and were then included in the intervention (18 patients). One control discontinued the previous intervention because of pregnancy and one due to lack of time. These two patients received instructions to increase their level of physical activity at their last visit. In the study group 30 patients were married or cohabitant and 9 were single. Seventeen patients (44%) stated that they had a physically demanding work. The median follow-up time was 5.2 years and the total range of time between baseline and follow-up was 3.8-6.2 years.

Pharmacological treatment and other IBS related interventions

Between the end of the previous intervention and the follow-up seven of the included patients participated in one or two other IBS related interventions. Four patients participated in a short IBS school, three sessions[18]. Three patients underwent treatment with nurse-administered gut-directed hypnotherapy. One of these patients also had one consultation visit to a dietitian. Medications that were used by the patients only at the follow-up and not at the start of the previous study are presented in Table 1.

IBS symptoms

The IBS-score (IBS-SSS) improved significantly as illustrated in Figure 2. Fifty-four percent of the patients had an improvement of more than 50 points, which is considered a clinically significant improvement[5]. Ten percent of the patients had their symptoms worsened with more than 50 points according to the IBS-score. The extra colonic score showed no significant changes (Figure 2).

A separate analysis of the IBS-SSS in the 24 [19 women, median age 50.5 (30.5-62.5) years] patients who had had no change in medication and had received no other IBS related intervention detected also an improvement in IBS-SSS, IBS-score 282 (184-379) *vs* 218 (85-342), $P = 0.002$. There was no significant change in the Extra colonic score.

The stool consistency had become firmer, from 4.5 (2.3-5.8) at baseline to 3.8 (1.5-4.8) at follow-up, $P = 0.004$. There was no significant change in the stool frequency per week at baseline 11 (5-21) compared with follow-up 9 (4-25), P = NS.

Psychological symptoms

The group demonstrated low levels of anxiety and depression in HADS at baseline, these low levels were reduced further at follow-up, and this was true for both

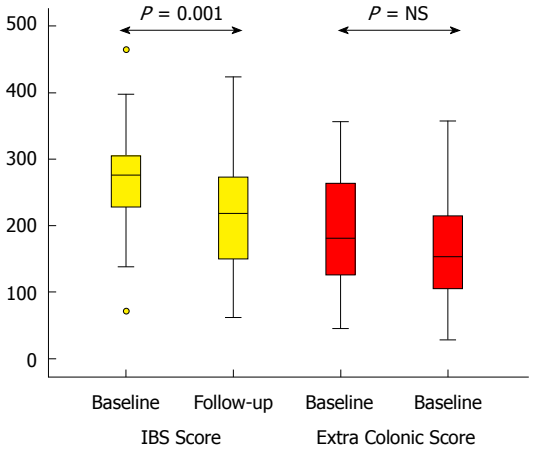

Figure 2 Irritable bowel syndrome severity scoring system at baseline and follow-up. IBS: Irritable bowel syndrome; NS: Not significant.

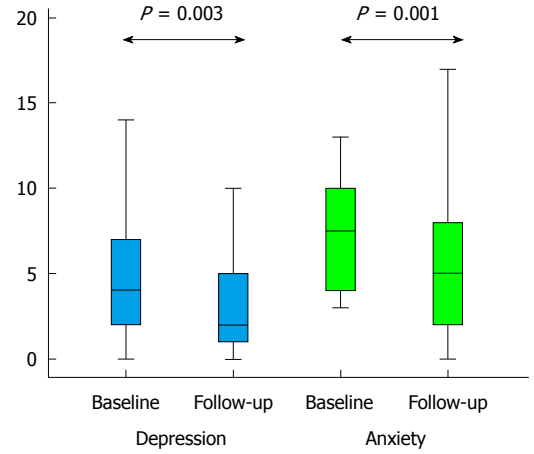

Figure 3 Hospital anxiety and depression scale at baseline and follow-up.

the depression and the anxiety subscale (Figure 3). At baseline 10 patients had depressive symptoms and 20 patients had anxiety as indicated by their scores. At follow-up this was reduced to 3 patients with depressive symptoms and 8 patients with anxiety.

There were significant improvements in five out of nine dimensions in the disease specific quality of life (IBS-QOL) (Table 2). Improved quality of life was also demonstrated on the subscales general health perceptions, emotional role and mental health in the SF-36 (Table 3).

Fatigue was significantly reduced according to two out of three subscales on the fatigue impact scale, namely the physical and the cognitive subscale, whereas there was no change on the psychosocial subscale (Figure 4).

Physical activity and weight

There was no significant difference in the oxygen uptake 31.8 (19.7-45.8) mL/min per kg at baseline and 34.6 (19.0-54.6) mL/min per kg at follow-up, *P* value is not significant. In the training diary the patients reported 3.2 (0.0-10.0) h of physical activity during the week before the baseline visit and 5.2 (0.0-15.0) h of physical activity during the week before the follow-up visit, $P = 0.019$. The range of intensity was rated from 9, very light exertion to 18, very hard exertion. The most common activity was walking followed by aerobics and cycling. The body weight had increased significantly from 66.6 (53.7-97.9) kg at baseline to 73.3 (52.6-95.7) kg at follow-up, $P = 0.037$.

Table 2 Irritable bowel syndrome quality of life at baseline and at follow-up

Dimension	Baseline	Follow-up	*P* value
Emotional functioning	56 (31-88)	69 (44-100)	0.001
Mental health	80 (50-100)	90 (45-100)	NS
Sleep	67 (33-100)	83 (42-100)	0.008
Energy	50 (38-88)	75 (50-100)	0.005
Physical functioning	67 (33-100)	92 (49-100)	0.002
Diet	60 (47-87)	73 (40-93)	NS
Social role	69 (43-94)	81 (50-100)	0.009
Physical role	69 (31-100)	81 (38-100)	NS
Sexual relations	60 (18-80)	60 (20-80)	NS

Values are median (10^{th}-90^{th} percentile). NS: Not significant.

Table 3 Short form 36 at baseline and at follow-up

Subscale	Baseline	Follow-up	*P* value
Physical functioning	90 (65-100)	95 (64-100)	NS
Physical role	50 (0-100)	100 (0-100)	NS
Bodily pain	51 (31-84)	51 (22-100)	NS
General health perceptions	54 (25-83)	67 (32-97)	0.006
Vitality	45 (20-80)	55 (25-85)	NS
Social functioning	75 (38-100)	75 (49-100)	NS
Emotional role	67 (0-100)	100 (0-100)	0.027
Mental health	72 (32-92)	74 (52-96)	0.016

Values are median (10^{th}-90^{th} percentile). NS: Not significant.

Changes between the end of the previous intervention and follow-up

This analysis includes 33 patients [26 women, median age 47 (28-62) years] who participated in the total 12 wk of intervention and completed the last visit in the first study. Significant improvements were detected on HADS on both subscales between the end of intervention and the follow-up. When the intervention was ended the results of the depression subscale was 5 (1-9) compared with 2 (0-7) at follow-up, $P = 0.004$. For anxiety the results were 8 (1.4-12.6) at end of intervention and 5 (1-11) at follow-up, $P = 0.004$.

The HRQOL was improved in four subscales of SF-36 namely bodily pain, general health perceptions, vitality and mental health (Table 4).

The stool consistency had become firmer, from 4.4 (3.1-5.5) at the end of intervention to 3.8 (1.8-4.8) at follow-up, $P = 0.001$. The body weight increased significantly from 66.3 (51.8-97) kg at end of intervention to 72.9 (53.2-96.5) kg at follow-up, $P = 0.015$. No other significant changes were observed on the other parameters (data not shown).

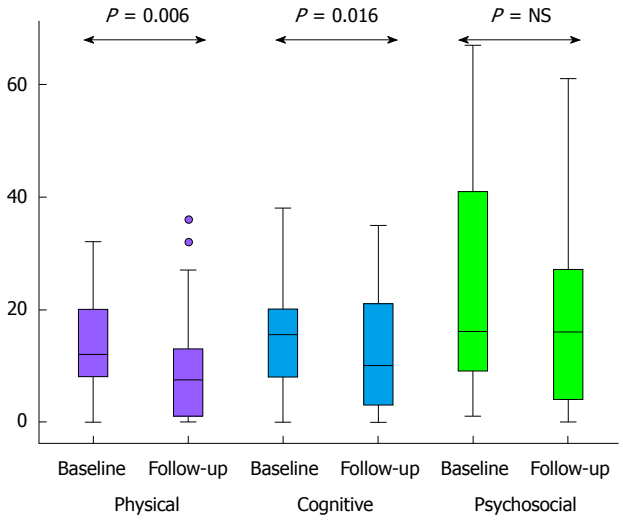

Figure 4 Fatigue impact scale at baseline and follow-up. NS: Not significant.

DISCUSSION

In a previous study the novel finding was described that a moderate increase in physical activity improves IBS symptoms and some aspects of the disease specific quality of life. However, there were no significant effects on fatigue, depression or anxiety directly after a 12 wk increase of physical activity[1]. The present work demonstrates that a 12 wk intervention followed by a continued moderate increase in physical activity, shows long term positive effects on IBS symptoms, quality of life, fatigue, anxiety and depression.

Our results are encouraging and confirm that a moderate increase of physical activity could be included in the initial management of patients with IBS. The mechanisms behind the improvement are probably complex and are not merely related to the level of maximum oxygen uptake. The maximal oxygen uptake declines with about 1% per year in normally active people[19]. The findings of maintained oxygen uptake and increased duration of physical activity per week in our group after 5 years is positive. The results are in contrast to results from patients with obesity, diabetes mellitus type 2 and hypertension showing that these groups are not easily motivated to increase and maintain their physical activity over a long period of time[2-4]. The reason for IBS patients to maintain a relatively stable oxygen uptake after 5 years can be due to the increase in the duration of physical activity per week. We may speculate that IBS patients may be well motivated to internalize and adopt the acquired experience of feeling better when they are physically active. The discomfort or pain these patients otherwise experience may enhance their motivation. Being physically active in order to improve the disease may also generate a feeling of control over the disease. A questionnaire study has shown a higher acceptance for lifestyle changes among IBS patients younger than 55 years[20]. Our patient group had a median age of 45 years and may easier adapt to lifestyle changes compared with elderly patients, which may be an additional explanation behind our results.

Table 4 Short form 36 at the end of intervention and at follow-up

Subscale	End of intervention	Follow-up	*P* value
Physical function	90 (47-100)	95 (56-100)	NS
Physical role	75 (0-100)	100 (0-100)	NS
Bodily pain	51 (26-72)	61 (22-100)	0.030
General health perceptions	60 (22-85)	63 (30-97)	0.013
Vitality	45 (15-78)	55 (22-83)	0.012
Social function	75 (43-100)	88 (50-100)	NS
Emotional role	100 (0-100)	100 (0-100)	NS
Mental health	64 (35-92)	76 (52-94)	0.006

Values are median (10^{th}-90^{th} percentile). NS: Not significant.

There are probably multiple mechanisms involved in the improvement of symptoms. Both physical factors as well as psychological factors are likely to play a role. The change of gas transit and colonic transit due to increased physical activity may contribute to these improvements. Villoria *et al*[21] showed that mild physical activity enhances intestinal gas clearance and reduces symptoms in patients complaining of abdominal bloating. The same group[22] showed earlier that physical activity improves gas transit as well as abdominal distension in healthy subjects, but not the perception of bloating. This may contribute to the improvement of symptoms shown in the present study. The improvement in patients with constipation-predominant IBS can be due to the positive effects of physical activity on symptoms of constipation[23,24].

The present work shows that 54% of the patients had a clinically significant improvement of the IBS symptoms compared with 43% in the previous study after a 12 wk intervention. This implies that the effects of physical activity cannot be explained only by a placebo effect. A pure placebo effect should have declined at the time of follow-up. However, a placebo effect is probably also involved. Brain-gut interactions may play a role in the processes leading to our results. Stress induces exaggeration of the neuroendocrine response and visceral perceptual alterations[25]. Physical activity counteracts the effects of stress and can therefore favourably influence brain plasticity[26]. General effects seem to be detected first after a longer period of increased physical activity and may explain the improvements in fatigue, depression and anxiety and quality of life. Factors like high levels of illness behavior, anxiety, sleep problems, and somatic symptoms are independent predictors of IBS onset[27]. Physical activity has a protective effect on depressive symptoms and may therefore protect against IBS symptom deterioration[1,28].

Our data also demonstrate a significant improvement in five aspects of the disease specific quality of life. In the previous study only two aspects of the IBS-QOL were improved after a 12 wk intervention. Earlier studies have shown that long term physical activity is an important determinant of health related quality of life in women[29].

Therefore it is important to consider the aspect of time when studies to assess the effect of physical activity on HRQOL are designed.

The physical and cognitive aspects of fatigue were improved in this study while fatigue was not affected in the previous study. Chronic fatigue and IBS often occur together[30] and fatigue is a symptom that is difficult to influence in other chronic diseases like liver disease[31]. Therefore the finding that fatigue in IBS can be affected by increased physical activity on the long-term is important. According to the review article by Puetz[32], there is an agreement among studies suggesting a strong, dose-response relationship between physical activity and the reduction of feelings of low energy and fatigue. This knowledge as well as our data may be used to motivate patients suffering from fatigue to be more active.

Clinically significant improvement was shown in depression and anxiety although the group had low scores at baseline. Studies on depression have previously demonstrated reduced symptoms secondary to increased physical activity[28]. The data presented in this study confirms previous data and reveals that a time with increased physical activity longer than 12 wk is needed to improve low levels of anxiety and depression[29].

One clear limitation in our study is that we do not have a control group in this part of the study. This is due to ethical concerns expressed by the ethics committee. The original design of the previous study was an intervention of 12 mo to improve physical activity. The committee did not accept a longer intervention than 12 wk because the control group would wait too long for the intervention.

Thirty-nine patients out of 76 were assessed on the follow-up after about five years. Given the long time to follow-up and given the fact that the patients were not compensated for loss of income during the visit, this is an expected proportion of patients. Fifty one percent have therefore participated. A larger proportion would have strengthen the study even more. However, the majority of patients not participating gave plausible reasons not to participate. The general impression when the patients were contacted was that their participation was related to their practical ability to participate and not to positive or negative experience on physical activity in IBS. One included patient expressed spontaneously the impression that physical activity was not effective for her and she stated that this was the reason for her to participate. A negative impression from the previous interventions seems not to stop the patients from participating.

There are some studies addressing the long term natural history of IBS or functional gastrointestinal disorders. Halder *et al*[33] studied the long term changes in functional gastrointestinal disorders and noticed that the prevalence is stable over time but there was a turnover of symptoms over time rather than total symptom resolution. Agréus *et al*[34] conducted a population based study demonstrating that more than 50% of the patients with IBS reported the same symptom profile after one and seven years. Symptom resolution in IBS was not observed in these studies indicating that physical activity in the present study had a positive influence on the course of the disease.

One of the findings in this study is that patients with IBS included in the previous study on increased physical activity had maintained an increased level of physical activity at follow-up. The patients reported a longer duration of physical activities in the training diary during the week before the follow up visit compared with baseline. This reflects the ambition of these patients to be physically active. We hypothesized that the increase of the physical activity would increase the oxygen uptake. At the time of the follow-up the most common activity was walking. Walking is in most cases not challenging enough to increase cardiorespiratory fitness. When designing future studies we would consider using a pedometer as a complement to the ergometer cycle test to measure physical activity. There is also a need of more studies on physical activity in IBS investigating the effect of different types of activities. Duration, intensity and frequency of the physical activity also have to be analyzed with a dose-response perspective considering the fact that endurance athletes report GI symptoms[35-37].

An important observation in the present study is the wide spectrum of positive clinical effects shown after a long time of follow up of about 5 years, which is unusual for interventional studies.

In conclusion, an intervention to increase physical activity improves IBS symptoms, as well as different aspects of the disease specific quality of life, fatigue, depression and anxiety on the long term. The present study supports the evidence for the positive effects of physical activity in IBS and defends physical activity as a treatment option for IBS. However, further research and larger studies are needed to elucidate the effects of different physical activities in the IBS subgroups.

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COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disease. There is no overall treatment for IBS know today. In a recent randomized controlled study moderately increased physical activity for 12 wk was tested as an intervention in IBS. Improvements in IBS symptoms as well as in some aspects of the disease specific quality of life were observed in the intervention group but not in the control group.

Research frontiers

In IBS several other non-pharmacological treatments has been studied such as patient education, hypnotherapy and other psychological treatments. These treatments often show good results however they are not available to the majority of patients.

Innovations and breakthroughs

A moderate increase of physical activity has been shown to improve gastrointestinal symptoms in IBS. This long-term follow-up shows that there is

an enhanced improvement about 5 years after participating in an intervention to increase physical activity. At follow-up there was an improvement in IBS symptoms, quality of life, fatigue, depression and anxiety, some of these improvements were not as evident in the previous study.

Applications

The study supports the evidence for the positive effects of physical activity in IBS and defends physical activity as a treatment option for IBS. Advice on increased physical activity can be given to patients with IBS in both primary care and secondary care.

Terminology

IBS is a very common functional bowel disorder. The patients suffer from diarrhea, constipation or both. Abdominal pain or discomfort and bloating are common symptoms of IBS. Other symptoms as impaired quality of life, depression and fatigue can accompany the gastrointestinal symptoms. Physical activity is used in medical care in both treatment and prevention of diseases. Physical activity has been found to improve a wide range of diseases like fibromyalgia, depression, hypertension and diabetes mellitus.

Peer review

The physical activity intervention is manageable in a clinical setting in both primary and secondary care, with low costs and a low risk of potential harmful effects. The authors have chosen an adequate set of questionnaires to evaluate their participants.

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ORIGINAL ARTICLE

Clinical Trials Study

Intraoperative endoscopic retrograde cholangio-pancreatography: A useful tool in the hands of the hepatobiliary surgeon

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Abstract

AIM: To evaluate the efficacy of intraoperative endoscopic retrograde cholangio-pancreatography (ERCP) combined with laparoscopic cholecystectomy (LC) for patients with gall bladder stones (GS) and common bile duct stones (CBDS).

METHODS: Patients treated for GS with CBDS were included. LC and intraoperative transcystic cholangiogram (TCC) were performed in most of the cases. Intraoperative ERCP was done for cases with proven CBDS.

RESULTS: Eighty patients who had GS with CBDS were included. LC was successful in all cases. Intraoperative TCC revealed passed CBD stones in 4 cases so intraoperative ERCP was performed only in 76 patients. Intraoperative ERCP showed dilated CBD with stones in 64 cases (84.2%) where removal of stones were successful; passed stones in 6 cases (7.9%); short lower end stricture with small stones present in two cases (2.6%) which were treated by removal of stones with stent insertion; long stricture lower 1/3 CBD in one case (1.3%) which was treated by open hepaticojejunostomy; and one case (1.3%) was proved to be ampullary carcinoma and whipple's operation was scheduled.

CONCLUSION: The hepatobiliary surgeon should be trained on ERCP as the third hand to expand his field of therapeutic options.

Key words: Obstructive jaundice; Endoscopic retrograde cholangio-pancreatography; Gall stones

Core tip: The incidence of common bile duct stones (CBDS) in patents with gall bladder stones (GS) varies between 7% and 20%. Management of CBDS is

changing with advances in endoscopic techniques in many regards. Laparoscopic cholecystectomy is the gold standard in treating GS. This has created controversies in the management of CBDS. The hepatobiliary surgeon should be trained in endoscopic retrograde cholangio-pancreatography as the third hand to expand his field of therapeutic options.

El Nakeeb A, Sultan AM, Hamdy E, El Hanafy E, Atef E, Salah T, El Geidie AA, Kandil T, El Shobari M, El Ebidy G. Intraoperative endoscopic retrograde cholangio-pancreatography: A useful tool in the hands of the hepatobiliary surgeon. *World J Gastroenterol* 2015; 21(2): 609-615 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/609.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.609

INTRODUCTION

The incidence of common bile duct stones (CBDS) in patients with gall bladder stones (GS) is 7 to 20%[1,2]. Laparoscopic cholecystectomy (LC) is the gold standard in treating GS (3). This has produced debates in the treatment of CBDS[3,4]. Many authors have tried to find an optimal option to manage combined CBDS and GS. Different options are available. All CBDS existing at the time of cholecystectomy should be extracted, since residual stones in the common bile duct (CBD) may cause subsequent hepatobiliary and pancreatic complications[4].

There are different options that exist for removal of CBDS, including preoperative endoscopic retrograde cholangiopan-creatography (ERCP) before LC, laparoscopic common bile duct exploration, open CBD exploration and postoperative ERCP[2,5,6]. The use of preoperative ERCP has been argumentative[4]. Many studies found that 40%-90% performed a useless procedure due to passed stones, failed clearance of CBD, and retained stones despite its complications[7-13]. Postoperative ERCP avoids unnecessary examination but has a failure rate of 7%-14%[2,8,10]. LC with intraoperative ERCP and endoscopic sphincterotomy (ES) is an alternative technique for management of GS and CBDS[2,11,14].

Laparoscopic common bile duct exploration (LCBDE) is successful in removing the CBD stones in 80%-95% of cases but it is time consuming with a morbidity rate about 4%-16%[15,16]. However, the limitation of this technique is when there are multiple large or impacted ductal stones. It needs more laparoscopic skill and a longer learning curve is required for LCBDE[17-21].

ERCP is done mainly by physicians and radiologists, and rarely by surgeons. The wide use of laparoscopic surgery and the advances in technology and training made ERCP and intraoperative US important aids in the hands of experienced hepatobiliary surgeons[1-3]. The optimal treatment of CBDS is dependent on the skills of the surgical team and the availability of instruments and endoscopies at the hospital. The single procedure has advantages over the two step procedure[2].

This study was planned to evaluate intraoperative ERCP combined with LC for patients with GS and CBDS as regards the success rate and safety, and to show the importance of ERCP for the hepatobiliary surgeon.

MATERIALS AND METHODS

Patients

Consecutive patients with GS and CBDS at the Gastroenterology Surgical Center, Mansoura University, Mansoura, Egypt, during the period from August 2011 through April 2013, were managed by a single step treatment combining laparoscopic cholecystectomy and intraoperative ERCP. After completion of LC in the same set, exclusion criteria included age older than 80 years, pregnancy, previous history of gastrectomy, or coagulopathy.

Informed consent was obtained from all patients to be included in the study, after explanation of the nature of the disease and possible treatment. The study was approved by the local ethical committee of our hospital.

Study procedure

All patients were subjected to careful history taking, clinical examination, and laboratory investigation including total serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT). Abdominal ultrasound was done for all cases to assess GB and CBD dilation and stones. Magnetic resonance cholangiography (MRCP) was done in some cases where US could not confirm the diagnosis. A preoperative and intraoperative prophylactic antibiotic (cefotax 1 g, iv) was given to all patients. Somatostatin was given routinely to all patients one hour before surgery (1 mL, sc) to prevent development of post ERCP pancreatitis.

Laparoscopic cholecystectomy was started in all cases using the standard 4-ports technique under general anesthesia. Intraoperative cholangiogram was performed by the transcystic route in all cases. When laparoscopic cholecystectomy was finished, we temporarily put in an intraabdominal drain and closed the ports and prepared for ERCP. ERCP was performed in the supine position or the semiprone position. Endoscopic sphincterotomy was done in all cases using an endoflex type double lumen sphincterotomy and both Terumo and Hydrajag wires. Removal of stones was done by balloon, dormia basket or mechanical lithotripsy in difficult cases. A completion cholangiogram was done to confirm freedom of the CBD from stones. Electrocautery probes were used to ensure haemostasis if there was suspicion of bleeding from the papillotomy.

Assessment

The following items were recorded: difficulty of cannulation of the biliary tract using Freeman score [1 = one to five attempts (easy), 2 = six to 15 attempts (moderate difficult), 3 = more than 15 attempts (difficult), 4 = failed][22]; the need for precut for cannulation; total

Table 1 Demographic data

Variables	Data
Age (yr)	32 (18-59)
Sex	
Male	20 (25)
Female	60 (75)
Bilirubin (mg)	3.8 (0.4-10)
AST (IU)	99 (21-468)
ALT (IU)	93 (24-1000)
CBD diameter (mm)	11 (6-15)
Size of stones in CBD	
Mean size (mm)	8 (5-17)

Data are expressed as absolute numbers (percentage) or median (range). CBD: Common bile duct stone.

Table 2 Results

Variables	Data
Duration of operation (min)	95 (75-200)
Hospital stay (h)	19 (18-24)
Time to reach papilla (s)	25 (20-45)
ERCP done	76 (95)
ERCP not done (passed stone by TCC)	4 (5)
Difficulty in cannulation	
Grade 1	50/76 (65.8)
Grade 2	24/76 (31.58)
Failure of cannulation	2 (2.6)
Precut papillotomy	8/76 (10.52)
Results	
ERCP with stone extraction	64 (84.2)
Passed stone	6 (7.9)
Short stricture lower end CBD with stone ES with stent	2 (2.6)
Long stricture lower 1/3 CBD...treated by open hepaticojejunostomy	1 (1.3)
Filling defect at the lower end biopsied... adenocarcinoma	1 (1.3)
Failure of cannulation	2 (2.6)
Method of stone extraction	
Balloon	52/66 (78.8)
Basket	4/66 (6.1)
Combined basket and balloon	8/66 (12.1)
lithotripsy	2/66 (3.1)

Data are expressed as absolute numbers (percentage) or median (range). ERCP; Endoscopic retrograde cholangio-pancreatography; CBD: Common bile duct stone; TCC: Transcystic cholangiogram.

operative time; hospital stay; and cost. Any complications of the procedure were assessed using Cotton's criteria: mild (2-3 d spent in hospital); moderate (4-10 d in hospital; or serious (more than 10 d in hospital or need surgical or radiological interference)[23].

Follow up

The patients were followed up on postoperative day 7, and then at 1 mo and 3 mo after the operation. Patients were also seen at our clinic if they developed symptoms between follow-up visits. Follow up was done by clinical examination, serum bilirubin and abdominal ultrasound.

Statistical analysis

Statistical analysis of the data in this study was performed using SPSS software, version 17. For continuous variables, descriptive statistics were calculated and were reported as median. Categorical variables were described using frequency distributions

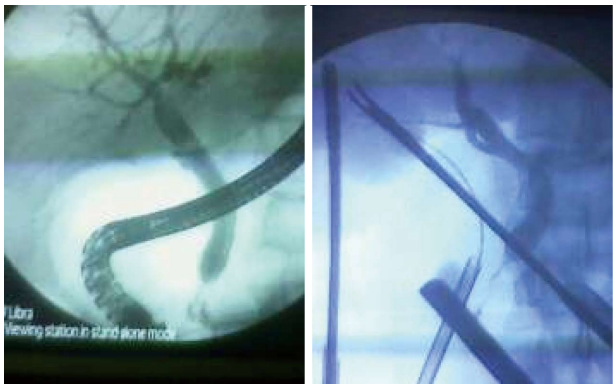

Figure 1 Short stricture at lower third common bile duct stone with small stone above the stricture.

RESULTS

Preoperative data

Eighty patients seen during the recruiting period, (20 men and 60 women) were eligible and entered the study. The median age was 32 years. All patients presented with abdominal pain and jaundice. The median preoperative bilirubin was 3.8 (range 0.4-10) mg (Table 1).

Abdominal ultrasound revealed the presence of multiple GS in all patients. The median diameter of the CBD was 11 (6-15) mm (Table 1).

Operative data

Laparoscopic cholecystectomy was successful in 79/80 cases (98.8%). Intraoperative transcystic cholangiogram (IOTC) was performed in 78 cases. IOTC was not performed in two cases due to failure of cannulation of the narrow cystic duct. It showed passed stones in four cases so ERCP was unnecessary for these cases.

ERCP was performed in 76 cases and was successful in 74 cases (97.3%). Failure of cannulation occurred in two cases (one case due to atrophic papilla, and another one due to juxta-diverticulum papilla). The cannulation was easy in 50/76 cases (65.8%) while precut was needed in 8 cases (10.5%) (Table 2).

Intraoperative ERCP showed a dilated CBD with stones in 64 cases (84.2%) where removal of stones were successful. Six cases (7.9%) showed passed stones. A short lower end stricture with small stones presented in 2 cases (2.6%) who were treated by removal of stones with stent insertion and follow up CT (Figure 1). Postoperative CT was free and removal of the stent was done 1 mo later. Long stricture lower 1/3 CBD was found in 1 case (1.3%) and treated by open hepaticojejunostomy (Figure 2), and 1 case (1.3%) was proved to be ampullary carcinoma and whipple's operation was scheduled (Figure 3, Table 2). Failure of cannulation occurred in 2 cases, (1 case due to atrophic papilla, and another case due to

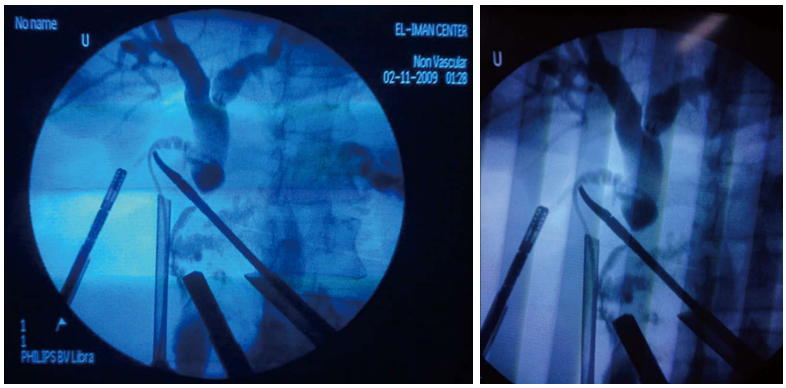

Figure 2 Long stricture at lower third common bile duct stone (Open hepaticojejunostomy was done).

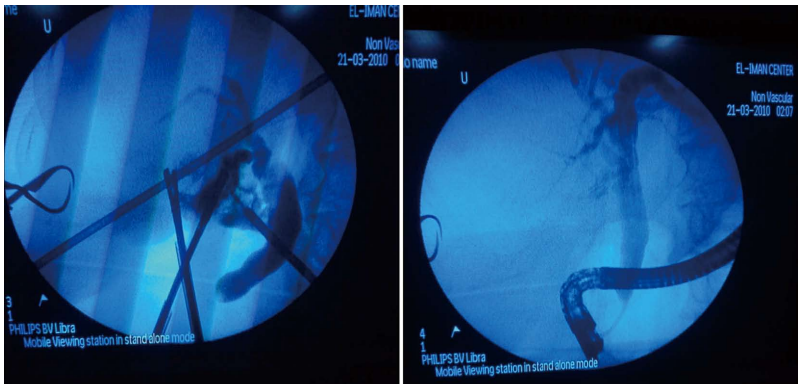

Figure 3 Filling defect in lower part of common bile duct stone. Biopsied-Ampullary carcinoma.

juxta-diverticulum papilla) and the decision was made to complete the two cases by laparoscopic CBD exploration.

Stone extraction was done by balloon in 56/66 cases (78.8%), by basket in 4/66 cases (6.1%), by combined balloon and basket in 8/66 cases (12.1%), and mechanical lithotripsy was needed in 2 cases (3.1%) that had large hard stones.

The median operative time was calculated for both maneuvers and found to be 95 (75-200) min. The postoperative course was smooth in all cases with no mortality. Melena occurred in 1 case due to peptic ulcer.

Postoperative data

The complication rate after ERCP was 5/76 (6.6%) and took the form of bleeding papillotomy in 2 cases managed by side view endoscopy using electrocautery probe, hyperamylasemia in 2 cases and passed conservatively, and pancreatitis in 1 patient which was managed conservatively

The median hospital stay was 19 (18-24) h. The average net cost was $850 ($700-1350) for both maneuvers (Table 2).

Follow up

All patients were followed up on postoperative day 7, 1 mo, and 3 mo postoperatively. No recurrent or residual stones were detected at these times.

DISCUSSION

The most popular methods of detecting CBDS include intraoperative cholangiography (IOC), intraoperative ultrasonography, ERCP, endoscopic ultrasonography, and MRC[4]. ERCP had its beginning in the late 1960s, with its introduction in the United States by McCune[24]. Endoscopic papillotomy and stone removal is successful in more than 90% of cases, with 5% morbidity and less than 1% mortality rate in expert hands[25]. Preoperative ERCP and ES is an effective option for removing CBDS in most cases, but only 10%-60% of patients will have stones on ERCP[1,6,8,9,26]. Even with strict selection criteria more than 10% of the preoperative ERCP are normal, and the possibility of occurrence of post-ERCP pancreatitis varies between 1%-13.5%[10,27-30]. Post-operative ERCP avoids undue trial but has a failure rate of 7%-14%[2,8,10].

Up till now ERCP has been mastered by either physicians or radiologists. The hepatobiliary surgical teams in our center have mastered ERCP, with more than 7000 procedures performed since 1991. So, we continuously have the availability to perform intraoperative ERCP (IO-ERCP) maneuvers.

ElGeidie *et al*[11] reported that LC and IO-ERCP is an available treatment for managing CBDS but it needs a well equipped hospital as regards endoscopies and a

skillful surgical team. The success rate for IO-ERCP was 97.8%, the mean surgical time was 112 min, postoperative stay was 1.3 d and the morbidity rate was 4.5%.

LC and IO-ERCP is a single procedure for management of CBDS that decreases the hospital stay and costs. It also reduces unnecessary ERCP and decreases the need to return to the operating room following technical failure of ERCP. Ghazal *et al*[2] performed a study of 45 cases with GS and with a suspected or confirmed CBDS. Cholecystectomy was completed laparoscopically in 44 patients. The conversion rate was 1/45 due to marked adhesions at the Calot's triangle. IOC was done in all cases and revealed CBDS in 36 cases. IO-ERCP with ES was performed successfully in 73.2% of patients. The mean surgical time was 119 min (ranging from 100 to 150 min). There was no postoperative morbidity related to the procedure and no evidence of retained CBDS on follow up[2].

DePalma *et al*[31] reported a 100% success rate in stone clearance with IO-ERCP in 15 cases, with a mean surgical time of 97.7 ± 30.4 min[31,32]. However, it needs organization to overcome the technical problems and to encourage the spread of this single procedure LC/ERCP[33]. It is difficult to assure the immediate availability of an endoscopist if stones are detected unexpectedly on IOC. This situation is time consuming and prolongs the operative times. The technical difficulties of the combined approach are related to both the supine position as more experience is required for successful cannulation of the papilla, and insufflation of gases[33].

In the present study the authors are surgeons who are experienced in both laparoscopy and ERCP; thus it is easy to secure an endoscopist if stones are detected unexpectedly on IOC immediately so the operative time in our study was shorter than other studies. Patients in our study were discharged after a mean hospital stay of 19 h (range 18-120 h). Williams and Vellacot[8] reported a hospital stay of 2.5 d, ranging from 1 to 5 d. Ghazal *et al*[2] reported that the mean hospital stay was 2.55 ± 0.89 d.

In our study some settings had been performed to solve the difficulties of IO-ERCP. It is technically more difficult to do ERCP in the supine position but it improves fluoroscopic visualization of hilar anatomy. We found that the position of the endoscope required for facing the papilla in the duodenum was somewhat different and was overcome by using specific techniques, such as clockwise rotation of the head of the endoscope or clockwise body rotation. To overcome the issue of intestinal distension, we perform LC and IOC first then after completion of LC we perform ERCP.

Single-stage LC/ERCP provides effective management for CBDS and may be helpful in selected patients who may not afford a second anesthetic maneuver[7]. Also, to increase the success rate of IO-ERCP, various rendezvous techniques were developed through the cystic duct[34]. Rabago *et al*[35] presented a prospective randomized study of LC with two different approaches: preoperative ERCP *vs* IO-ERCP for CBDS. Intraoperative ERCP was performed using a rendezvous technique.

A Swedish registry shows successful bile duct cannulation was achieved in 92% of the ERCPs performed. The presence of CBDS was seen in 36.8% of examinations. Perioperative and postoperative morbidities were 2.5% and 9.8%, respectively. The rate of ERCP-induced pancreatitis was 2.7%, and the hospital mortality rate was 5.9%[36].

In conclusion, hepatobiliary surgery is an expanding field of surgery. The hepatobiliary surgeon should be acquainted with operative ultrasound, endoscopy and ERCP as the third hand to expand his field of therapeutic options. This will limit the time loss in difficult biliary situations.

COMMENTS

Background

Surgeons have tried to find an optimal option to manage combined common bile duct (CBD) stones and gall bladder stones (GS). Different options are available. All CBD stones (CBDS) present at the time of cholecystectomy should be removed, since residual stones in the CBD may cause subsequent hepatobiliary and pancreatic complications. There are different options exist for removal of CBDS, including preoperative endoscopic retrograde cholangio-pancreatography (ERCP) before laparoscopic cholecystectomy (LC), laparoscopic common bile duct exploration, open CBD exploration and postoperative ERCP.

Research frontiers

ERCP is performed mainly by physicians and radiologists, and rarely by surgeons. The wide use of laparoscopic surgery and the advances in technology and training make ERCP and intraoperative US important aids in the hands of the experienced hepatobiliary surgeon.

Innovations and breakthroughs

The optimal treatment of CBDS is dependent on the skills of the surgical team and availability of instruments and endoscopies at the hospital. There is no doubt that the single procedure has advantages over the two step procedure. This study was planned to evaluate intraoperative ERCP combined with LC for patients with GS and CBDS as regards the success rate and safety, and to show the importance of ERCP for the hepatobiliary surgeon.

Applications

The hepatobiliary surgeon should be trained in ERCP as the third hand to expand his field of therapeutic options.

Peer review

This manuscript of "Intraoperative endoscopic retrograde cholangio-pancreatography, a useful tool in hands of hepatobiliary surgeon" is well written.

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ORIGINAL ARTICLE

Clinical Trials Study

Copy number variations are progressively associated with the pathogenesis of colorectal cancer in ulcerative colitis

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Abstract

AIM: To evaluate the association of known copy number variations (CNVs) in ulcerative colitis (UC) progressing to colorectal cancer.

METHODS: Microsatellite instability analysis using the National Cancer Institute's panel of markers, and CNV association studies using Agilent 2 × 105 k arrays were done in tissue samples from four patient groups with UC: those at low risk (LR) or high risk of developing colorectal cancer, those with premalignant dysplastic lesions, and those with colitis-associated colorectal cancer (CAC). DNA from tissue samples of these groups were independently hybridized on arrays and analyzed. The data obtained were further subjected to downstream bioinformatics enrichment analysis to examine the correlation with CAC progression.

RESULTS: Microarray analysis highlighted a progressive increase in the total number of CNVs [LR (n = 178) *vs* CAC (n = 958), 5.3-fold], gains and losses [LR (n = 37 and 141) *vs* CAC (n = 495 and 463), 13.4- and 3.3-fold, respectively], size [LR (964.2 kb) *vs* CAC (10540 kb), 10.9-fold] and the number of genes in such regions [LR (n = 119) *vs* CAC (n = 455), 3.8-fold]. Chromosome-wise analysis of CNVs also showed an increase in the number of CNVs across each chromosome. There were 38 genes common to all four groups in the study; 13 of these were common to cancer genes from the Genetic Disease Association dataset. The gene set enrichment analysis and ontology analysis highlighted many cancer-associated genes. All the samples in the different groups

were microsatellite stable.

CONCLUSION: Increasing numbers of CNVs are associated with the progression of UC to CAC, and warrant further detailed exploration.

Key words: Ulcerative colitis; Colorectal cancer; Molecular analysis; Microsatellite instability; Copy number variations

Core tip: Ulcerative colitis (UC) confers an increased risk of colorectal cancer (CRC). The role of copy number variations (CNVs) in different cancers including sporadic CRC has been established but their association in the development of colitis-associated neoplasia is not well described. Reports to date are limited to only a particular stage (*e.g.*, dysplasia or cancer) in the development of colitis-associated cancer. In this first study of its kind, we report the association of increased numbers of known CNVs with the progression of UC to colitis-associated cancer.

Shivakumar BM, Rotti H, Vasudevan TG, Balakrishnan A, Chakrabarty S, Bhat G, Rao L, Pai CG, Satyamoorthy K. Copy number variations are progressively associated with the pathogenesis of colorectal cancer in ulcerative colitis. *World J Gastroenterol* 2015; 21(2): 616-622 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/616.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.616

INTRODUCTION

Longstanding ulcerative colitis (UC) confers an increased risk of colorectal cancer (CRC)[1-6]. The frequency of colitis-associated colorectal cancer (CAC) in the Asia-Pacific region has been variously reported to be similar to or lower than that reported from the West, mostly based on retrospective studies[6-12]. Recent data from this low prevalence region suggest that premalignant lesions may not be uncommon in patients with longstanding UC, if dysplasia is methodically looked for[13].

The inflammation-dysplasia-carcinoma sequence defines carcinogenesis in UC and is accompanied by a series of molecular changes[14]. The major molecular pathways in the development of CRC involve chromosomal instability (CIN) and microsatellite instability (MSI), which are associated with an increase in the range of gene expression and phenotypic changes[15,16]. Studies have found that around 80% of the tumors with MSI have a near-diploid karyotype and a distinct genetic alteration distinguishable from those of microsatellite stable cancers[17].

Copy number variations (CNVs), a source of genetic diversity in humans under CIN and affecting gene dosage, are also believed to play a major role in human health and disease. CNVs can lead to altered expression of genes thereby contributing to cancer development. Profiling these can help in identifying tumor suppressor genes and oncogenes[18]. In the past decade, studies have established that even though common CNVs with low penetrance levels contribute only minimally or modestly to the causation of cancer, their collective impact on the predisposition to cancer must be considered while estimating the cancer risk[19]. CNVs constitute important genetic changes in various cancers including sporadic CRC, but their association with neoplasia in UC is not well described[20-23].

Copy number alterations detected by array-based Comparative Genomic Hybridization (aCGH) can be directly related to discovery of the underlying genes and/or molecular mechanisms involved with tumorigenesis, especially so with high or moderate penetrant CNVs[24,25]. Such discovery of altered regions associated with cancer may help in classifying the cancer patient at the molecular level along with the clinico-pathological features. With this background, the present study was aimed at elucidating the CNVs associated with the pathogenesis of CAC, a complex disease.

MATERIALS AND METHODS

Ethics

This study was approved by the Ethics Committee of Kasturba Hospital, Manipal. All the patients provided informed consent before participation.

Patient tissue and DNA Extraction

Patients with UC were recruited into 4 groups: UC-low risk (LR): UC patients with disease duration less than 7 years; UC-high risk (HR): UC patients with disease duration more than 7 years in case of extensive colitis or more than 10 years for left sided colitis; UC-premalignant (PM): UC patients who had any type of dysplasia (low grade or high grade); and UC-CAC: UC patients who were found to have cancer. Fresh biopsy specimens were immediately digested with proteinase K (0.1 mg/mL) in the presence of 1% sodium dodecyl sulphate (Sigma-Aldrich, United States). DNA was extracted using phenol-chloroform, followed by ethanol precipitation. DNA was checked for purity and stored at -20 ℃ until further analysis.

Microsatellite instability analysis

MSI status was examined using 5 microsatellite markers [National Cancer Institute (NCI), Bethesda Panel]. The assay was carried out using appropriate primer sequences and the corresponding fluorescent dyes and polymerase chain reaction as described elsewhere[26].

2 × 105 k CNV association microarray analysis

DNA from appropriate colonic tissue samples in these groups were independently hybridized on 2 × 105 k CNV association microarray slides (Agilent Techn-

Table 1 Characteristics of colitis-associated colorectal cancer association analysis using 2 × 105 k in different groups of samples during the progression of ulcerative colitis with respect to copy number variations

	UC-LR	UC-HR	UC-PM	CAC
Total number of CNVs	178	271	616	958
Number of CNVs with gain	37	190	465	495
Number of CNVs with loss	141	81	151	463
Overall CNV coverage (in kb)	964.2	2368.5	4875	10540.1
Number of genes within CNVs	119	141	318	455

CNVs: Copy number variations; UC: Ulcerative colitis; CAC: Colitis-associated colorectal cancer; LR: Low risk; HR: High risk; PM: Premalignant.

ologies, CA, United States) and analyzed according to the manufacturer's protocol. Briefly, genomic DNA samples were sheared using a cycle of 15 s "on" and 15 s "off" for 15 min in an ultrasonic processor (Thomas Scientific, NJ, United States) with a 2 mm probe with amplitude set at 40. The purified sheared DNA samples were differentially labeled; test DNA (test genome) with fluorescent Cy5 and the pooled normal reference DNA (reference genome) with Cy3 dyes. Hybridization, washing and scanning of the arrays were performed according to the manufacturer's protocol. Feature extracted data were analyzed with Genomic Workbench v5.0 software (Agilent Technologies, CA, United States) using the ADM-2 aberration detection algorithm (threshold 5.0) and the log2 ratios (± 0.25) as cut-off values with genomic boundaries switched on as track file of 022837. All genomic data reported in the present study were based on NCBI build 36 (hg18) of the human genome[27].

Bioinformatics enrichment analysis

Bioinformatics scanning approaches such as DAVID, Gene Set Enrichment Analysis (GSEA), Genetic Disease Association dataset (GAD), *etc.*, were used to explore the significance of a large variety of biological mechanisms and functional importance including associations with various cancer datasets in order to find the important set of enriched genes with significant functions in developing CRC.

RESULTS

Patient sample details

Samples were included from the following patient groups: UC-LR: n = 20; 10 male, 10 female, median age: 42 years; UC-HR: n = 20; 10 male, 10 female, median age: 45 years; UC-PM: n = 6; 4 male, 2 female, median age: 41 years; UC-CAC: n = 2; 1 male, 1 female, median age: 38 years. Subjects undergoing colonoscopy and found to have a normal examination and normal histology (n = 20; 10 male, 10 female, median age: 49 years) were included as controls. There was no statistically significant difference between the different groups in terms of age and sex in the study.

Table 2 2 × 105 k CNV analysis of all the samples and gene list analysis using gene set enrichment analysis for functionally significant families of genes

	UC-LR	UC-HR	UC-PM	CAC
Cytokines and growth factors	0	0	2	2
Transcription factors	7	8	18	25
Cell differentiation markers	2	2	6	8
Protein kinases	7	8	9	8
Translocated cancer genes	1	4	5	11
Oncogenes	2	5	7	11
Tumor suppressor	0	0	0	1

UC: Ulcerative colitis; CAC: Colitis-associated colorectal cancer; LR: Low risk; HR: High risk; PM: Premalignant.

MSI analysis

Samples in all the groups did not show any instability in the microsatellites analyzed and all were microsatellite stable.

2 × 105 k CNV analysis

The number of CNV regions progressively increased by up to 5-fold with advancing stages of disease (LR to CAC): 178 in LR, 271 in HR, 616 in PM and 958 in CAC. CNV coverage (size) was found to increase 10-fold with progressive stages from LR (total of 964 kb) to CAC (10540 kb). While the number of CNV regions showing gains increased with the advancing stages of disease, regions showing loss did not follow any particular pattern (Table 1). The number of genes encompassed within the CNV regions in each group increased substantially from 119 (LR) to 455 (CAC).

The chromosome-wise distribution of gains and losses of CNVs also showed an increase in number and size with disease progression (Figure 1A and D). The average number of CNVs per chromosome was < 5 in LR, increasing to > 30 in CAC. In addition, only two chromosomes (1 and 6) had altered CNVs > 100 kb in length in LR, while in CAC all but chromosomes 14, 18 and 21 harbored CNVs > 100 kb in length. LR showed individual CNVs up to 5 kb in size, but in the premalignant and malignant samples most CNVs were above the 5 kb range (Figure 1B).

Enrichment of gene sets by bioinformatics

To gain further insight of these CNV regions and their functional significance, we analyzed chromosomal gains and losses across all 4 sample groups using various computational tools and databases. By using Venny tool analysis, 38 genes were found common to LR, HR, PM and CAC (Figure 1C). Common genes analysis using Venny for our 4 groups of genes with the reported human GAD genes specific to cancer yielded 13 genes. GSEA analysis for genes from the CNV regions showed an increase in the functionally significant families of genes, such as transcription factors, oncogenes and other cancer-related genes (Table 2). Gene ontology analysis also

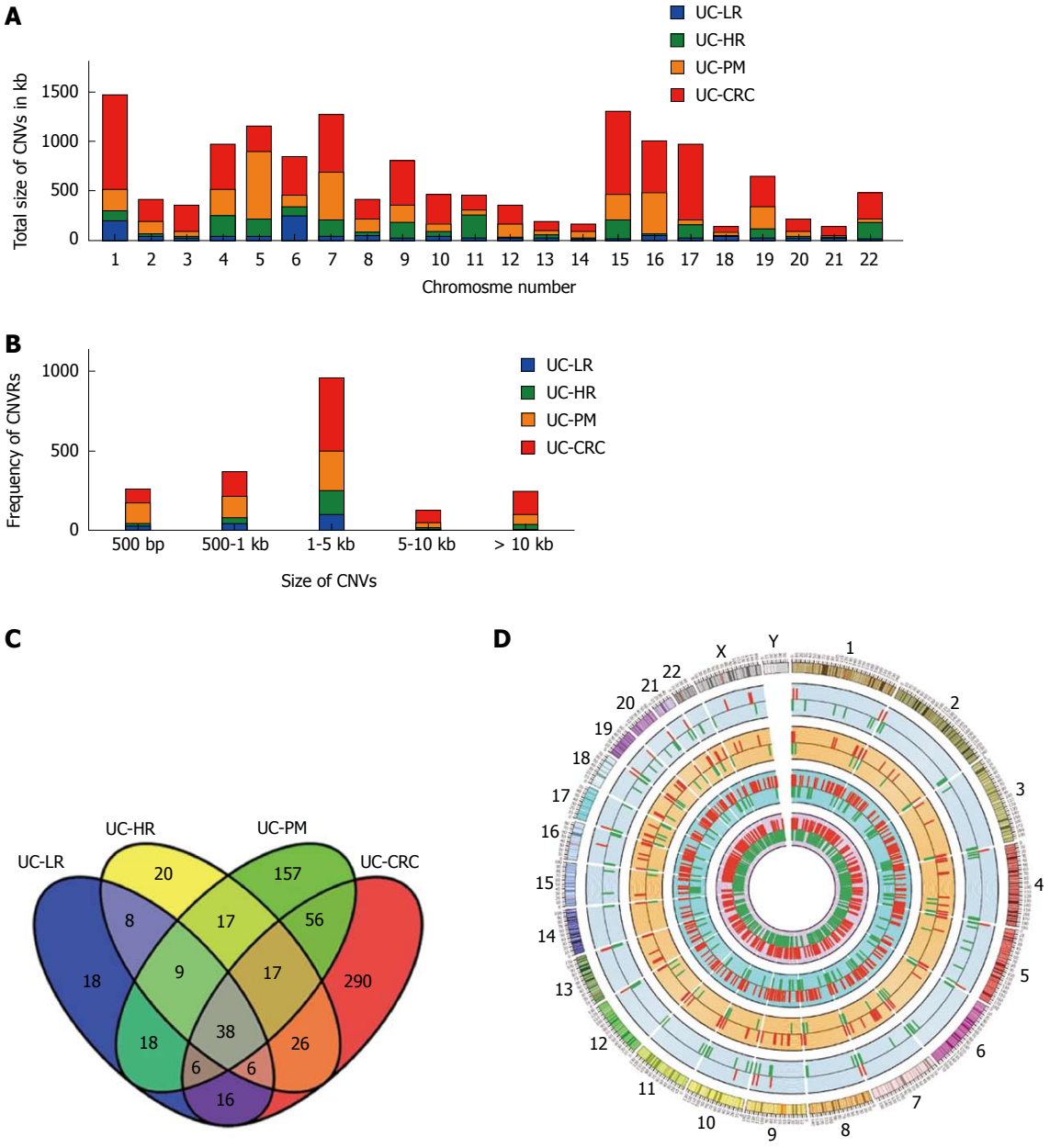

Figure 1 Size distribution and overlap analysis of copy number variable regions and associated genes. A: Each chromosome wide copy number variation (CNV) size distribution across samples from ulcerative colitis (UC) to colitis associated colorectal cancer (CAC); B: Histogram representation of distribution of CNV sizes on all 4 samples analyzed by CNV association microarrays; C: Venn diagram showing the number of unique and common genes in different groups from UC to CAC. D: CIRCOS plot highlighting progressive CNV association analysis during UC progression to CRC using 2×105 k arrays (UC-LR, UC-HR, UC-PM and UC-CRC or CAC: in the order from outer circle to inner circle). LR: Low risk; HR: High risk; PM: Premalignant.

showed an increased enrichment of the genes involved in extracellular biological processes among the 4 groups of samples (Figure 2).

DISCUSSION

The major aim of this study was to analyze the comprehensive association of known CNVs during various stages of UC progressing to CRC and thereby to understand the role of CNVs in inflammation-associated cancer development. The study, one of the first of its kind using 2 × 105 k CNV association arrays on DNA extracted from tissue samples has shown a progressive involvement of CNVs at different levels as the disease progresses from UC to CAC. Increasing numbers of CNVs were found to be associated with the progression of the disease from earlier stages to cancer. Other factors such as the size of CNVs and number of genes from these CNV regions were similarly found to be correlated with neoplastic progression. Bioinformatics enrichment analysis of CNV genes also enumerated putative functionally important cancer-associated genes. Hence, the study highlights the importance of classifying UC patients into subgroups at various stages of progression using clinical details in the evaluation of molecular pathomechanisms involved in CAC.

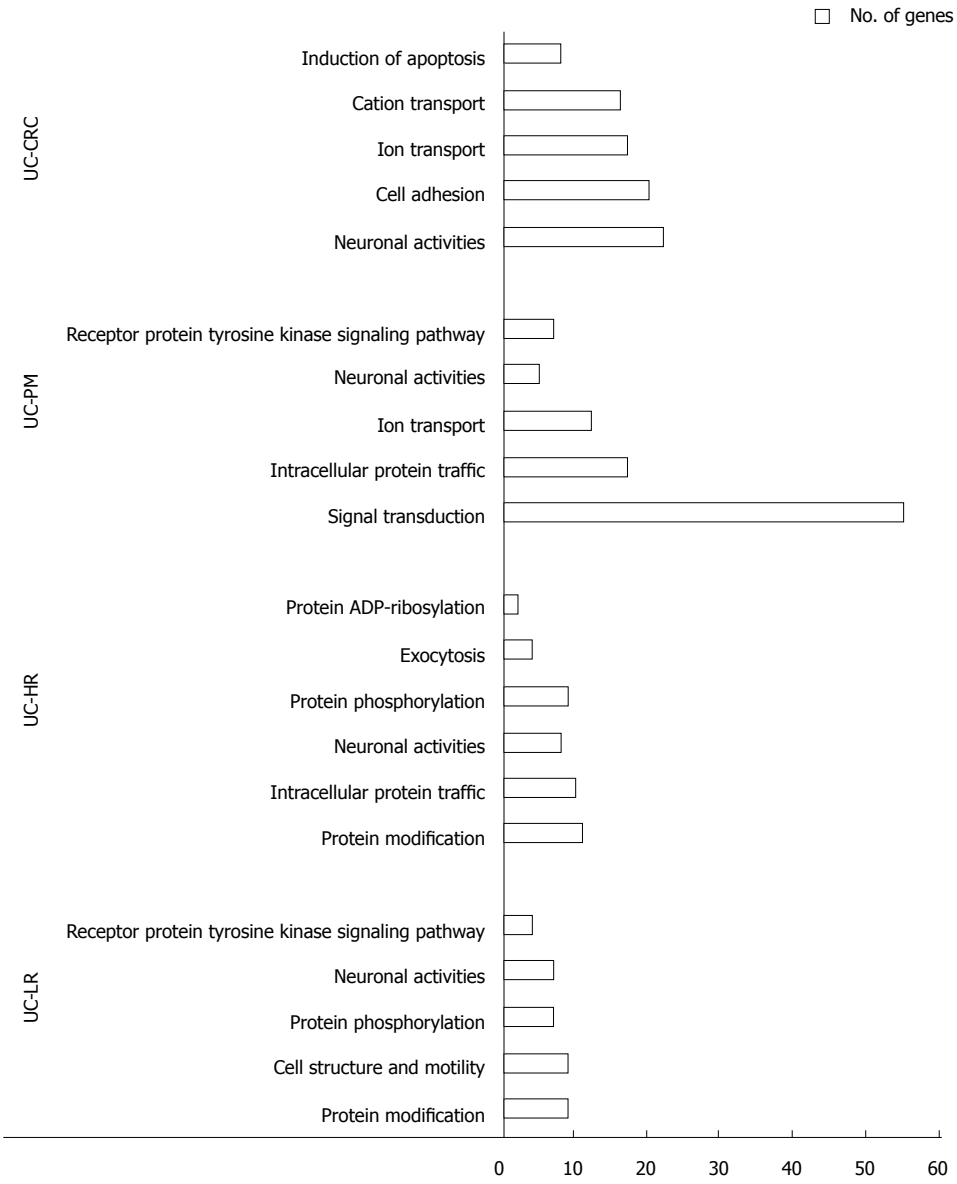

Figure 2 Summary results of gene ontology analysis carried out on 2 × 105 k data from different ulcerative colitis sample groups. X-axis: Molecular function and Y-axis: Number of genes involved. UC: Ulcerative colitis; CAC: Colitis-associated colorectal cancer; LR: Low risk; HR: High risk; PM: Premalignant.

The 2 × 105 k array has been successfully used in large sample-based studies for CNV association in common diseases[28]. A population-specific array (2 × 105 k CNV association array) with a target of analyzing the association of CNVs has shown promising results albeit with a limited number of inflammation-related and cancer-coordinating genes in the study. Being a tissue based study, the cells, and the CNVs therein being heterogeneous because of the pooling of the samples, could probably have affected the assessment of CNVs[29]. In this aspect, our results from tissue DNA samples have identified the CNV regions and the important genes situated in them that are associated with various stages of progression of UC to CAC. Unlike the present report, earlier studies on colitis-associated neoplasia used conventional methods (chromosomal genomic hybridization or bacterial artificial chromosome arrays) and were limited to only one stage of progression[21-25].

In another important observation, the genes encompassing these CNV regions matched with the cancer gene sets from various databases such as GAD or GSEA, and highlight the importance of these CNVs in carcinogenesis. Genes from amplified or gain CNV regions may act as oncogenes while the loss regions are likely to be embedded with tumor suppressor regions[18]. Gene ontology analysis further highlighted the significant number of genes involved in various molecular and biological functions from these CNV regions, increasing as the disease progressed to CAC. Thus, our data can also be used in future research to determine their definitive contribution to colorectal carcinogenesis, upon functional validation of genes from these CNV regions.

MSI is believed to play a role in the pathways of UC-associated and sporadic CRCs, contributing at a frequency of approximately 15%-20% compared to 80% CIN in the case of CRC[14]. The present study using the

NCI panel of Bethesda markers found no instability in any of the samples. One reason for this difference could be the use of a cancer-specific panel and recruiting only 2 patients with CAC in the study[26]. Reports suggest that CIN is greater in microsatellite stable samples[17,30].

The study of CNVs and cancer is in its infancy, but recent advancements in and the availability of technology is ensuring that more studies are being reported in this area. There is tremendous scope for further studies considering the effect of this form of genetic variation on cancer predisposition and the association with cancer genes. To our knowledge, this is the only study available till now on the association of CNVs with UC stratified into different stages of evolution to CAC. In a first study of its kind, using the association arrays of higher resolution on tissue samples we have demonstrated the progressive changes in CNVs as UC advances to cancer, establishing the importance of such genomic alterations in the pathogenesis of CAC. These results clearly indicate a major role for CNVs in the pathogenesis of CAC, warranting further focused studies on the regions and genes identified.

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COMMENTS

Background

Ulcerative colitis (UC) patients have a higher risk of developing cancer with increased duration of disease. The epidemiological data of colitis-associated colorectal cancer (CAC) in the Asia-Pacific region has been reported as being similar to or lower than that from the West. The accumulation of a series of molecular changes that accompany the progressive pathological changes in the inflammation-dysplasia-carcinoma sequence in UC has been well established. The role of copy number variations (CNVs) in different cancers including sporadic colorectal cancer (CRC) has been evaluated but their association with the stages of development CAC is not well described.

Research frontiers

CNVs in different cancers including sporadic CRC has been established but their association with the development of UC to CAC is not well described and previous studies are generally limited to a particular stage of disease. In this study, the authors have attempted to elucidate the association of CNVs with the pathogenesis of CAC carcinogenesis in a stage-wise manner for the first time.

Innovations and breakthroughs

Recent studies have highlighted the importance of structural variations such as CNVs in different cancers, including sporadic CRC, but there is a lack of similar types of studies in CAC. This is the only study to date that has stratified UC patients as low risk, high risk, premalignant and malignant for evaluating their CNV association. Progressive changes in CNVs are shown as UC advances to cancer establishing the importance of such genomic alterations in the pathogenesis of CAC.

Applications

By exploring and understanding these CNVs in the progression of CRC in UC through various stages, this study paves the way for future studies to evaluate the contribution of specific genes in colorectal carcinogenesis, with potential future possibilities for them as diagnostic markers and possible therapeutic targets.

Terminology

CNVs: Structural variations in the genome of approximately 1 kb or larger in size, including genomic imbalances such as amplifications and deletions, balanced translocations or inversions, are altogether universally referred to as CNVs. MSI: Microsatellites are repeated sequences of DNA and instability is the result of defective mismatch repair in the cells which is more commonly found in cancerous cells.

Peer review

In this study, the authors reported CNVs in the tissue samples from various stages of progression of UC through to CAC, and known CNVs were found to be increasingly associated with the progression of UC to CAC. Overall, these findings are well written with interests.

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ORIGINAL ARTICLE

Clinical Trials Study

Use of disposable graduated biopsy forceps improves accuracy of polyp size measurements during endoscopy

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Abstract

AIM: To determine the accuracy of endoscopic polyp size measurements using disposable graduated biopsy forceps (DGBF).

METHODS: Gradations accurate to 1 mm were assessed with the wire of disposable graduated biopsy forceps. When a polyp was noted, endoscopists determined the width of the polyp; then, the graduated biopsy forceps was inserted and the largest diameter of the tumor was measured. After excision, during surgery or endoscopy, the polyp was measured using the vernier caliper.

RESULTS: One hundred and thirty-three colorectal polyps from 119 patients were studied. The mean diameter, by post-polypectomy measurement, was 0.92 ± 0.69 cm; 83 were < 1 cm, 36 were between 1 and 2 cm, and 14 were > 2 cm. The mean diameter, by visual estimation, was 1.15 ± 0.88 cm; compared to the actual size measured using vernier calipers, the difference was statistically significant. The mean diameter measured using the DGBF was 0.93 ± 0.68 cm; compared to the actual size measured using vernier calipers, this difference was not statistically significant. The ratio between the mean size estimated by visual estimation and the actual size was significantly different from that between the mean size estimated using the DGBF and the actual size (1.26 ± 0.30 *vs* 1.02 ± 0.11).

CONCLUSION: The accuracy of polyp size estimation was low by visual assessment; however, it improved when the DGBF was used.

Key words: Disposable graduated biopsy forceps; Polyp size measurement; Colonoscopy; Accuracy

Core tip: In this study, we designed a disposable graduated biopsy forceps and used the forceps as "scale plate" to measure the polyp size. We enrolled 133 polyps from 119 patients and found that the accuracy of the visual estimation for a polyp size was low but could be improved if the disposable graduated biopsy forceps were used as a scale. Though some slight

deviation still existed for estimation of polyps over 2 cm, the difference was not significant and did not affect treatment.

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INTRODUCTION

The size of a tumor, detected at colonoscopy, is associated with the subsequent management of patients. If the size of a colon polyp is less than 3 cm, endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) could achieve complete resection with few complications[1,2]. If a colon polyp is greater than 3 cm, the bowel would need to be resected by endoscopic resection; these cases have been associated with a high risk of complications. However, for colon polyps less than 3 cm, the methods used and risks associated with endoscopic resection are different; that is, for polyps 2-3, 1-2 cm and those less than 1 cm[3,4]. Several clinical guidelines include the size and extent of the tumor as an important factor for deciding on the use of endoscopy for resection of colon polyps. Therefore, accurate measurement of colon polyps, during colonoscopy, is crucial for the appropriate management of patients with colon polyps[5-7]. However, there are no standard criteria for measuring the size of a colon polyp during colonoscopy.

Many endoscopists evaluate the size of a polyp based on their personal experience, which may be inconsistent with the actual size of the colon tumor. Eichenseer *et al*[8] compared the estimated size of 10 to 25-mm polyps, as determined during endoscopy, with the size determined by post-fixation histopathology of the polyps by 15 different endoscopists; they found that the mean size variation between the polyp size estimation at endoscopy and the size determined by histopathology of the polyps was 73.6% (range of mean size variation, 13%-127%). In addition, 62.6% (range, 0%-91%) included polyps that were clinically sized incorrectly; overestimation of the polyp size, during endoscopy, was more common than underestimation. Furthermore, some endoscopists inaccurately estimated the size of adenomas, and this led to inappropriate surveillance recommendations. In order to evaluate the differences between the size estimated by endoscopists and the actual size, as determined by histopathology, we developed a system using disposable graduated biopsy forceps to evaluate the size of colon polyps and compared this to both the estimation reported by endoscopists and the final size determined by histopathology.

MATERIALS AND METHODS

Ethics

The study was approved by the Ethics Committee of the Third Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. All patients signed an informed consent form.

Study subjects

The graduated biopsy forceps was developed based on the traditional disposable biopsy forceps. At the beginning of the steel wire of the traditional disposable biopsy forceps, gradations were drawn every 1 mm along a 3-cm total length, using medical pigment. This was used as a scale plate for estimating the size of the tumors. From April to September 2013, patients with polyps were enrolled from the National Center of Colorectal Surgery, Nanjing University of Traditional Chinese Medicine. First, when the endoscopists discovered a polyp or tumor, they assessed the largest diameter of the tumor; then, they inserted a graduated biopsy forceps and measured the largest diameter of the tumor from the vertical view; lastly, the "gold standard" for the largest diameter of the tumor was accurately measured using a vernier caliper, after excising the tumor by surgery or endoscopy (Figure 1). A correct measurement was defined as a variation of less than 10% between the size evaluated and the actual size as measured using a vernier caliper. The accuracy rate was defined as the number of polyps accurately evaluated divided by the total number of polyps. Five endoscopists who had an experience of over 2000 cases of colonoscopy were involved in the study.

Statistical analysis

Data were analyzed using SPSS 17.0 software for Windows. The paired Student's *t*-test was used to compare the ratio of the estimated size, by the endoscopists, to the actual size measured using the vernier caliper, with the ratio of the size measured using the graduated biopsy forceps to the actual size measured using the vernier caliper. Analysis of variance was used to compare the differences among the three groups. A P-value < 0.05 was considered statistically significant.

RESULTS

Basic clinical characteristics of the polyps

One hundred and thirty-three colon polyps from 119 patients (76 males and 43 females; average age, 58.29 ± 11.45 years; range, 28-82 years) were included. Among the polyps, 40 were rectal polyps, 43 sigmoid colon polyps, seven descending colon polyps, 12 transverse colon polyps, and 31 cecum or ascending colon polyps. Among the patients, two underwent laparoscopic colectomy, and all others had endoscopic mucosal resection. One hundred and seven were adenomas, while four were villous adenomas (of which, one was mucosal

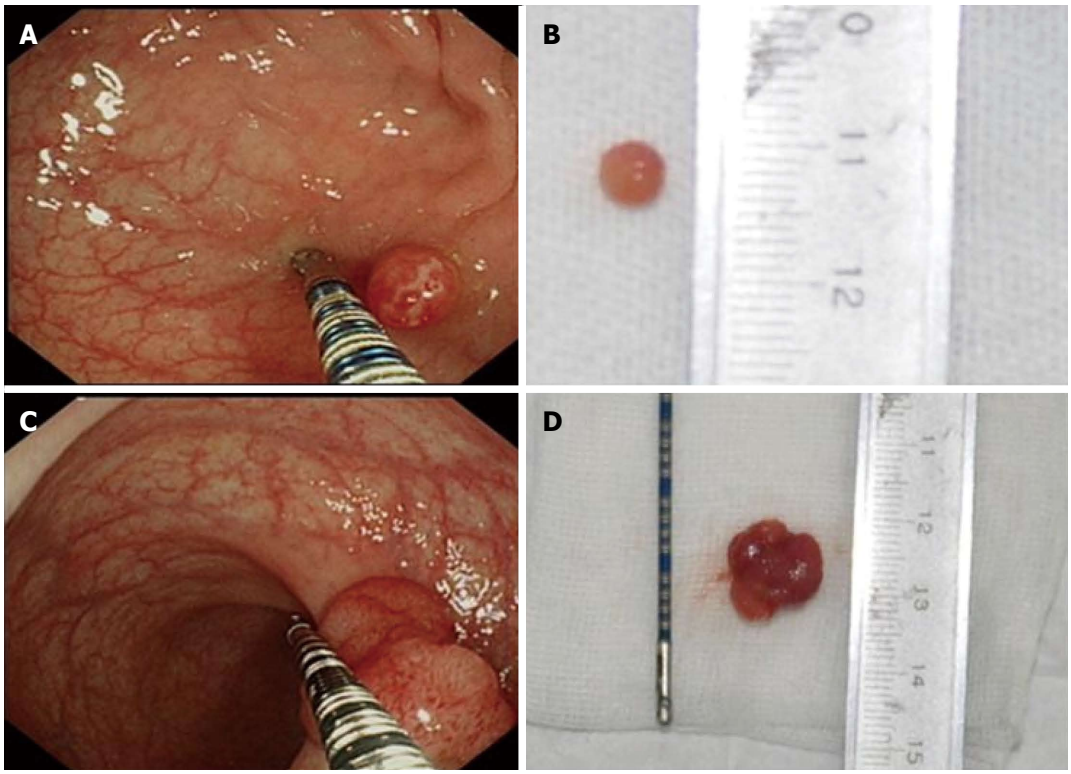

Figure 1 Comparison of measurements using the graduated biopsy forceps and the vernier caliper. A: 0.6 cm, measured using the graduated biopsy forceps; B: 0.6 cm, measured using the vernier caliper; C: 1.4 cm, measured using the graduated biopsy forceps; D: 1.4 cm, measured using the vernier caliper.

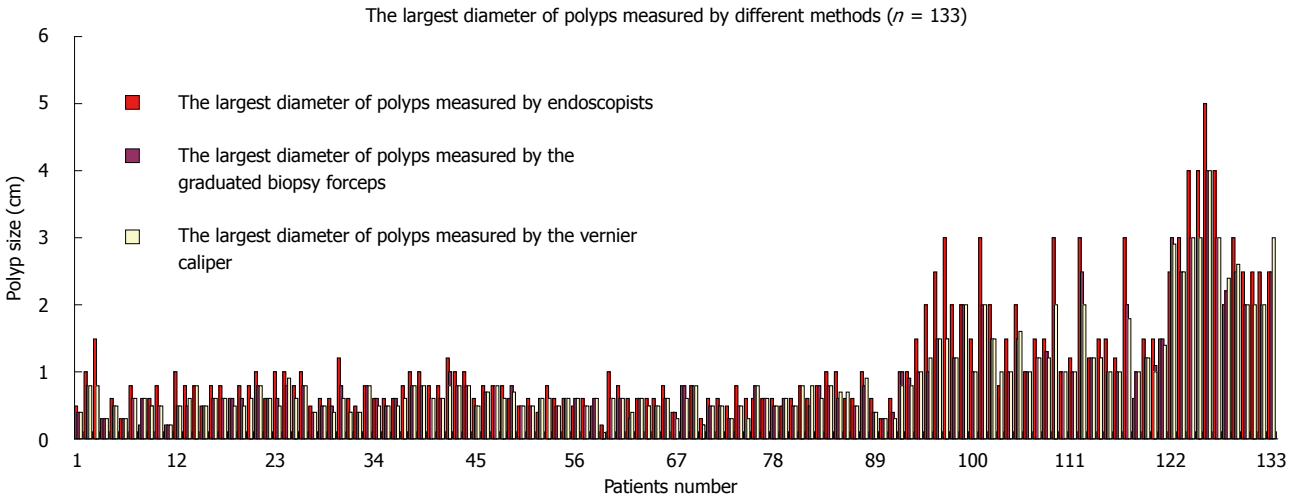

Figure 2 Comparison of the largest diameter measured using the three different methods.

cancer, two were tubulovillous adenomas and one was a neuroendocrine tumor, a carcinoid tumor).

Influence of different tumor sizes on tumor size evaluation

The mean largest diameter of the resected polyps, measured using a vernier caliper, was 0.92 ± 0.69 cm (range, 0.1-4.0 cm); among the polyps, 83 were < 1 cm, 36 were between 1 and 2 cm and 14 were > 2 cm (Figure 2).

For those polyps < 1 cm, the mean largest diameter evaluated by the endoscopists was 0.70 ± 0.23 cm (range, 0.2-1.5 cm); this was significantly different from the actual size measured using the vernier caliper (0.57 ± 0.17 cm) (P = 0.000). The mean largest diameter measured using the graduated biopsy forceps was 0.58 ± 0.17 cm; this was not significantly different from the actual size as measured using the vernier caliper (P = 0.096).

For polyps 1 to 2 cm, the mean largest diameter measured using the vernier caliper was 1.31 ± 0.34 cm, while the mean largest diameter by the endoscopists was 1.77 ± 0.71 cm (range, 0.6-3.0 cm); the difference was statistically significant (P = 0.000). The mean largest

diameter measured using the graduated biopsy forceps was 1.323 ± 0.43 cm (range, 0.9-2.1 cm); this was not significantly different from the mean largest diameter measured using the vernier caliper (P = 0.688).

For polyps that were > 2 cm, the mean largest diameter measured using the vernier caliper was 2.70 ± 0.58 cm, while the mean largest diameter evaluated by the endoscopists was 3.12 ± 0.91 cm (range, 2.0-5.0 cm); this difference was statistically significant (P = 0.006), which implied that the endoscopists tend to overestimate the polyp size. The mean largest diameter measured using the graduated biopsy forceps was 2.64 ± 0.59 cm; this was not significantly different from the mean largest diameter measured using the vernier caliper (P = 0.223).

Influence of different tumor sizes on the accuracy of tumor size estimation

For 83 polyps less than 1 cm, only 22.2% (8/36) could be accurately estimated by the endoscopists, while 86.7% (72/83) could be accurately measured using the graduated biopsy forceps; this difference was statistically significant (P = 0.000).

For 36 polyps between 1 and 2 cm, only 11.1% (4/36) could be accurately estimated by the endoscopists, while 66.7% (24/36) were accurately measured using the graduated biopsy forceps; this difference was statistically significant (P = 0.009).

For polyps over 2 cm, none were accurately estimated by endoscopist assessment, while 57.1% were accurately measured using the graduated biopsy forceps.

DISCUSSION

The size of a colon polyp, assessed at colonoscopy, is crucial for determining patient management. Many clinical guidelines use the size and extent of the tumor as an important factor for determining whether to resect colon polyps using endoscopy[5-7]. However, accurate estimation of the size of a polyp, during colonoscopy, is not guaranteed. Currently, the size of a polyp is evaluated by endoscopists based on their personal experience; however, there is great variation among endoscopists with regard to the evaluated size and actual size of a colon polyp. In some studies, the oncological potential of a colon polyp has been related to its size and shape, indicating that the measurement of the colon polyp size was an important factor for determination of the risk associated with the colon polyp[9,10]. In this study, the difference between the actual size of the colon polyp and the size assessed by the endoscopists was significant. The actual size measured using the vernier caliper varied from the size estimated by the endoscopists, and this difference was statistically significant, especially for polyps greater than 1 cm. The reason for the variation in size estimation *via* endoscopy was the absence of a "scale plate". All size estimations were done based on the endoscopists' experience. Those endoscopists who were more experienced could accurately evaluate the size of the colon polyps; however, the size estimated by those endoscopists who were less experienced varied from the actual size. The accuracy of size estimation was improved by using the graduated biopsy forceps during endoscopy[11]. Morales *et al*[12] used an open biopsy forceps as a guide to measuring the colon polyp size during colonoscopy; however, there were significant differences between the endoscopic estimates and the post-polypectomy measurements for three-quarters of the polyps. Gopalswamy *et al*[13] compared the accuracy of a linear probe, visual estimation and forceps for estimating polyp size during colonoscopy; they found that the measurement of the polyp size using a linear probe had the best agreement with the actual polyp size, followed closely by visual estimation. The open biopsy forceps method was the least accurate. However, the linear probe requires special software to estimate size, which adds to the examination time and cost of the procedure. In recent years, the computed tomography (CT) colonoscopy has been used to estimate the size of colon polyps with a high degree of accuracy. However, the patients required bowel preparation before a CT colonoscopy and the procedure was very costly[14-16].

To estimate the size of colon polyps, we developed a graduated biopsy forceps based on the traditional disposable biopsy forceps. Gradations were drawn every 1 mm along the 3-cm total length using medical pigment. The accuracy of estimating the size of the colon polyps increased when using the graduated biopsy forceps. In this study, the mean estimated size, by the endoscopists, was 1.15 cm; this was different from the actual size measured using the vernier caliper (0.92 cm) and the difference was statistically significant. However, the mean size measured using the graduated biopsy forceps was 1.02 cm, which was statistically similar to the actual size measured using the vernier caliper (0.93 cm). Therefore, the size measured using the graduated biopsy forceps was consistent with the actual size as measured using the vernier caliper. The ratio of the size estimated by the endoscopists to the actual size measured using the vernier caliper was 1.26, while the ratio of the size measured using the graduated biopsy forceps to the actual size measured using the vernier caliper was 1.02; the difference between these two ratios was statistically significant (P = 0.000). These findings show that the graduated biopsy forceps could improve the accuracy of size estimation of colon polyps.

Different polyp sizes affected the accuracy of size estimation. The bigger the tumor size was, the less accurately the tumor was estimated. From our study, the polyp size by endoscopist tends to be overestimated, and the polyp size tends to be more accurately estimated when we used the graduated biopsy forceps. For polyps less than 1 cm, 86.7% could be accurately measured. For polyps between 1 and 2 cm, only 11.1% could be accurately estimated by the endoscopists, while 66.7% were accurately measured using the graduated biopsy forceps. These findings indicate that using the graduated

biopsy forceps improved the accuracy of size estimation and decreased the variation of measurements.

However, this type of graduated biopsy forceps could only measure polyps along the longitudinal axis of the bowel; polyps that surrounded the enteric cavity could not be directly measured with the graduated biopsy forceps. In such cases, estimations by the endoscopists were needed; they identified polyps that surrounded the enteric cavity. In this study, for 14 polyps that were > 2 cm, variation existed in 42.9% (6/14) of the polyps, especially for the lateral spread of the polyps. The size measurement could not be determined in one view, and in such cases the assessment might be different from the actual size.

In conclusion, the estimation of colon polyp size during endoscopy based on endoscopists' experience had lower accuracy; use of the graduated biopsy forceps during endoscopy improved the accuracy of size estimation. Although there were variations in laterally spreading colon polyps that were > 2 cm, the variations were significantly decreased and they did not affect treatment and follow-up.

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COMMENTS

Background

The size of a tumor, detected at colonoscopy, is associated with the subsequent management of patients. However, there are no standard criteria for measuring the size of a colon polyp during colonoscopy. Several studies showed that some endoscopists inaccurately estimated the size of adenomas, and this led to inappropriate surveillance recommendations. Therefore, in order to evaluate the differences between the size estimated by endoscopists and the actual size, as determined by histopathology, the authors developed a system using disposable graduated biopsy forceps (DGBF) to evaluate the size of colon polyps and compared this to both the estimation reported by endoscopists and the final size determined by histopathology.

Research frontiers

In order to evaluate the differences between the size estimated by endoscopists and the actual size, as determined by histopathology, authors developed a system using disposable graduated biopsy forceps to evaluate the size of colon polyps and compared this to both the estimation reported by endoscopists and the final size determined by histopathology.

Innovations and breakthroughs

This paper showed an easy method to measure the size of colon polyps. The study showed that the accuracy of polyp size estimation was low by visual assessment; however, it improved when the DGBF was used. Although some slight deviations still existed for estimations of polyps over 2 cm, the difference was not significant.

Applications

The DGBF is a very easy method to accurately measure the size of polyps during endoscopy. It is a very useful method and does not increase the medical cost.

Peer review

The manuscript is novel and potentially helpful to the practicing gastroenterologist. It is a very interesting paper and presents an original view of the problem.

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ORIGINAL ARTICLE

Observational Study

Prevalence of *Helicobacter pylori* infection and atrophic gastritis in patients with dyspeptic symptoms in Myanmar

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Abstract

AIM: To survey the detailed analyses for *Helicobacter pylori* (*H. pylori*) infection and gastric mucosal status in Myanmar.

METHODS: A total of 252 volunteers with dyspeptic symptoms (155 female and 97 male; mean age of 43.6 ± 14.2 years) was participated in Yangon and Mandalay. The status of *H. pylori* infection was determined based on 5 different tests including rapid urease test, culture, histology, immunohistochemistry and serology. Histological scores were evaluated according to the update Sydney system and the Operative Link for Gastritis Assessment system. Pepsinogen (PG) Ⅰ and PG Ⅱ were measured using enzyme-linked immunosorbent assays.

RESULTS: The overall prevalence of *H. pylori* infection

was 48.0%. There was no relationship between age and infection rate. Even in young group (less than 29 years old), the *H. pylori* infection rate was relatively high (41.9%). The prevalence of *H. pylori* infection was significantly higher in Yangon than that of Mandalay. *H. pylori* infection was significantly associated with the presence of gastric mucosal atrophy. All 7 subjects with peptic ulcer were infected with *H. pylori*. Although *H. pylori*-positive subjects showed stronger gastritis than *H. pylori*-negative subjects, most cases had mild gastritis.

CONCLUSION: We revealed the prevalence of *H. pylori* infection in patients with dyspeptic symptoms in Myanmar. The *H. pylori* infection was a risk factor for peptic ulcer and stronger gastritis.

Key words: *Helicobacter pylori*; Myanmar; Pepsinogen; Atrophy

Core tip: The prevalence of *Helicobacter pylori* (*H. pylori*) infection in Myanmar has not been elucidated. Our study revealed that the overall prevalence of *H. pylori* infection was 48.0% in patients with dyspeptic symptoms. Even among young group (less than 29 years old), the *H. pylori* infection rate was relatively high (41.9%). Nevertheless, most cases showed mild gastritis, which suggests that the moderate of the incidence of gastric cancer might be attributed to the mild atrophy. All 7 subjects with peptic ulcer were infected with *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is strongly related with the development of gastroduodenal diseases including peptic ulcer and functional dyspepsia[1]. Although *H. pylori* infection is also a major factor to development of gastric cancer[2], the difference of *H. pylori* infection rate is not enough to explain the difference of the incidence of gastric cancer in the world. For example, despite the high prevalence of *H. pylori* infection in India, the incidence of gastric cancer in India is much lower than in other countries, the so-called Asian enigmas[3]. In addition to host and environmental factors, as a part, the difference of the incidence of gastric cancer irrespective of *H. pylori* infection rate can be explained by the difference of virulence factors of *H. pylori* rather than *H. pylori* infection rate[4]. In fact, *H. pylori* strains isolated in India are Western-type strains, on the other hand, those of Chinese are East-Asian-type strains[5,6]. In Thailand, *H. pylori* strains isolated from Chinese-Thai showed East-Asian-type whereas those from Thai-Thai showed Western-type strains[7].

Myanmar is located in Southeast Asia bordered by China, Thailand, India, Laos and Bangladesh. The age-standardized incidence rate (ASR) of gastric cancer in Myanmar was reported to be 11.2/100000 per year[8] (http://globocan.iarc.fr/), which is higher than that of India and Thailand, and lower than that of China (6.1, 3.1 and 22.7/100000, respectively). To our knowledge, there is no previous study published focusing on the *H. pylori* infection in Myanmar. To understand the reason for higher incidence of gastric cancer in Myanmar than India or Thailand, it is important to elucidate of *H. pylori* infection rate in Myanmar. In addition, phylogeographic analyses with genomic difference of *H. pylori* strains can assume the migration of human populations[9]. Therefore, analyses of *H. pylori* strains isolated from Myanmar might be contributed to the exploration of human migration pattern in south Asian countries.

Furthermore, the gastric cancer risk can be assessed by the status of gastric atrophy[10]. Not only endoscopic and histological examination but also the measurements of serum pepsinogen (PG) I and PG I / Ⅱ levels can be available to examine the status of gastric mucosal atrophy. A meta-analysis showed that a PG I level ≤ 70 ng/mL and a PG I / Ⅱ ratio ≤ 3 had a sensitivity of 57%, specificity of 80%, positive predictive value of 15%, and negative predictive value of 83% in screening for atrophic gastritis to detect gastric cancer[11]. However, the proper cut-off value can be various according to the geographic difference.

In this study, we first disclosed the infection rate of *H. pylori* in Myanmar by multiple tests including rapid urease test, culture, histology, immunohistochemistry and serology. In addition, we examined the status of gastric mucosa based on histology and serology.

MATERIALS AND METHODS

Study population

We consecutively recruited a total of 252 volunteers with dyspeptic symptoms (155 female and 97 male; mean age of 43.6 ± 14.2 years, range 13 to 85 years old) in our prospective study in 2012. The survey took place in the largest city, Yangon (n = 182) and the second largest city, Mandalay (n = 70). Subjects with a history of partial gastric resection were excluded. Total of 252 subjects were consisted of 43 at ≤ 29 years old, 65 at 30-39 years old, 56 at 40-49 years old, 55 at 50-59 years old, and 33 at ≥ 60 years old. Peripheral blood was collected from each subject after overnight fasting. Samples were collected into serum tubes and centrifuged within 1 h after collection. Separated sera were used for serological identification of *H. pylori* and measurement of the PG

levels. All reagents for *H. pylori* cultures (*e.g.*, disposable forceps, transport mediums) were brought from Thailand and Japan. We performed endoscopy on the same day with blood collection. Written informed consent was obtained from all participants, and the protocol was approved by the Ethics and Research Committee of University of Medicine (1), Myanmar, that of Mandalay General Hospital, that of Thammasat University Hospital as well as that of Oita University Faculty of Medicine, Japan.

During each endoscopy session, 4 gastric biopsy specimens were obtained (three from the lesser curvature of the antrum approximately three cm from the pyloric ring and one from the greater curvature of the corpus). Three specimens from the antrum were used for *H. pylori* culture, rapid urease test and histological examination. One specimen from the corpus was used for histological examination. Peptic ulcers and gastric cancer were identified by endoscopy. Gastritis was defined as *H. pylori* gastritis in the absence of peptic ulcer or gastric malignancy.

Status of H. pylori infection

To maximize the diagnostic accuracy, 5 different methods were combined for the diagnosis of *H. pylori* infection including rapid urease test, culture, histology, immunohistochemistry, and serology. Subjects were considered to be *H. pylori*-negative when all 5 tests were negative, whereas *H. pylori*-positive status required at least one positive test result.

H. pylori culture

One biopsy specimen from the antrum was homogenized in saline and inoculated onto Mueller Hinton Ⅱ Agar medium (Becton Dickinson, NJ, United States) supplemented with 7% horse blood without antibiotics. The plates were incubated for up to 10 days at 37 ℃ under microaerophilic conditions (10% O_2, 5% CO_2 and 85% N_2). *H. pylori* was identified on the basis of colony morphology, Gram staining and positive reactions for oxidase, catalase, and urease. Isolated strains were stored at -80 ℃ in Brucella Broth (Difco, NJ, United States) containing 10% dimethylsulfoxide and 10% horse serum. For histology, all biopsy materials were fixed in 10% buffered formalin for 24 h, and then embedded in paraffin. Serial sections were stained with hematoxylin and eosin and with May-Giemsa stain. The degree of bacterial load was classified into four grades: 0, "normal"; 1, "mild"; 2, "moderate"; and 3, "marked" according to the updated Sydney system[12]. More than or equal of 1 grade of bacterial load was defined as *H. pylori* positive.

Serological analysis of H. pylori infection and PG

Anti-*H. pylori* IgG levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Eiken Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Serum PG Ⅰ and PG Ⅱ levels were measured using Pepsinogen ELISA (Eiken, Co. Ltd.) according to the manufacturer's instructions. Individuals with a serum *H. pylori* antibody titer ≥ 10 U/mL were classified as *H. pylori*-positive according to the manufacturer's instructions; those with PG Ⅰ levels ≤ 70 ng/mL and a PG Ⅰ/Ⅱ ratio ≤ 3.0 were classified as PG-positive according to the Japanese guidelines[13].

Immunohistochemistry

Immunohistochemistry was performed as described previously[14]. Briefly, after antigen retrieval and inactivation of endogenous peroxidase activity, tissue sections were incubated with α-*H. pylori* antibody (DAKO, Denmark) overnight at 4 ℃. After washing, the sections were incubated with biotinylated goat antirabbit IgG (Nichirei Co., Japan), followed by incubation with a solution of avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA, United States). Peroxidase activity was detected using H_2O_2/diaminobenzidine substrate solution. For all cases, we performed Giemsa staining using a serial section to identify the presence of *H. pylori*. If the *H. pylori* identified by Giemsa staining was found to be positively immunostained, we judged the case as positive.

Staging for gastritis

The degree of gastritis was classified using 4 grades: 0, normal; 1, mild; 2, moderate; and 3, marked according to the updated Sydney system[12]; samples of grade 1 or more were considered atrophy-positive according to a previous report[15]. In addition, on the basis of the topographic locations (antrum and corpus), the gastritis stage (the severity and topography of atrophy) was assessed according to the Operative Link on Gastritis Assessment (OLGA) system[16,17].

Statistical analysis

Data were analyzed using SPSS, version 19 (SPSS Inc., Chicago, IL, United States). Statistical evaluation was performed by the χ^2 test to compare discrete variables and the Mann-Whitney *U*-test and the *t*-test to compare continuous variables. Differences in prevalence in each group were analyzed using the Mantel-Haenszel method. Spearman rank coefficients (*r*) were determined to evaluate the association between the severity of mucosal atrophy and PGs. Multiple backward stepwise logistic regression analyses were performed to examine the associations of atrophy with the main predictor variables, such as age, sex, *H. pylori* infection. For each variable, the OR and 95%CI were calculated. A two-tailed *P* value < 0.05 was considered significant. Receiver operating curves (ROC) were used to calculate the best cut-off values for discriminating atrophic gastritis by PG Ⅰ/Ⅱ.

RESULTS

Prevalence of H. pylori infection in Myanmar

Table 1 showed *H. pylori* positive rate in each test. The results of histology and immunohistochemistry were identical. Among 5 tests, serological test showed higher

Table 1 Prevalence of *Helicobacter pylori* infection in each diagnostic test *n* (%)

	Age (yr)					
	-29	30-39	40-49	50-59	60-	Total
n	43	65	56	55	33	252
Serum	16	27	21	21	8	93
	(37.2)	(41.5)	(37.5)	(38.2)	(24.2)	(36.9)
RUT	9	33	17	21	6	86
	(20.9)	(50.8)	(30.4)	(38.2)	(18.2)	(34.1)
Culture	9	26	18	18	3	74
	(20.9)	(40.0)	(32.1)	(32.7)	(9.1)	(29.4)
Histology	11	29	20	23	7	90
	(25.6)	(44.6)	(35.7)	(41.8)	(21.2)	(35.7)
IHC	11	29	20	23	7	90
	(25.6)	(44.6)	(35.7)	(41.8)	(21.2)	(35.7)
Final	18	37	26	28	12	121
	(41.9)	(56.9)	(46.4)	(50.9)	(36.4)	(48.0)

RUT: Rapid urease test; IHC: Immunohistochemistry.

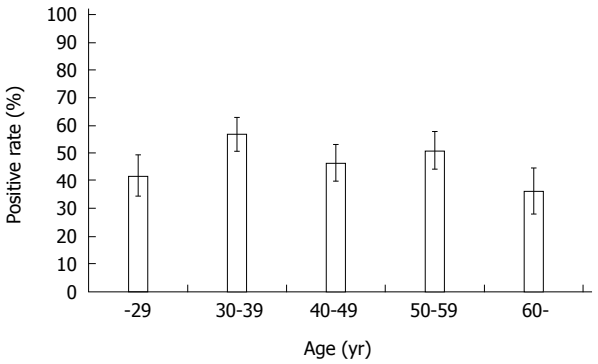

Figure 1 Prevalence of *Helicobacter pylori* infection by age group in Myanmar. *Helicobacter pylori* (*H. pylori*) infection was examined by 5 different methods including rapid urease test, culture, histology, immunohistochemistry, and serology. Subjects were considered to be *H. pylori*-negative when all 5 tests were negative, whereas *H. pylori*-positive status required at least one positive test result. Each bar shows the percentage of positive cases and the standard error.

positive rate compared with culture, although it did not reach a statistical significance ($P = 0.07$). When subjects were considered to be *H. pylori* positive in case at least one test showed positive, overall, the prevalence of *H. pylori* infection in Myanmar was 48.0% (121/252). Figure 1 shows the prevalence of *H. pylori* infection according to various range age groups. There was no statistical difference in the positive rate with age ($P = 0.31$). Even in younger age group, the prevalence of *H. pylori* infection was more than 40%. There was no difference of *H. pylori* infection rate between male and female ($P = 0.43$).

The prevalence of *H. pylori* infection differed among the 2 cities. The prevalence of *H. pylori* infection in Yangon was 41.9% (13/31) at ≤ 29 years old, 62.2% (28/45) at 30-39 years old, 55.8% (24/43) at 40-49 years old, 51.2% (21/41) at 50-59 years old, and 40.9% (9/22) at ≥ 60 years old. On the other hand, the prevalence of *H. pylori* infection in Mandalay was 41.7% (5/12) at ≤ 29 years old, 45.0% (9/20) at 30-39 years old, 15.4% (2/13) at 40-49 years old, 50.0% (7/14) at 50-59 years old, and 27.3% (3/11) at ≥ 60 years old. The overall prevalence of *H. pylori* infection in Yangon was significantly higher than that of Mandalay even when the age was adjusted by the Mantel-Haenszel method (52.2% *vs* 37.1%, $P = 0.04$).

Endoscopic findings and H. pylori infection rate

In endoscopic diagnosis, gastritis was most common findings (233/252, 92.4%). Gastric and duodenal ulcer was found at 3 cases (1.1%) and 4 cases (1.5%), respectively. Gastric cancer was found in 3 cases (1.1%). Other diagnosis including submucosal tumor was found in 9 subjects. Among 233 subjects with gastritis, 109 (46.8%) were infected with *H. pylori*. On the other hand, all 7 subjects with peptic ulcer were infected with *H. pylori*, which was significantly higher than that of gastritis (100 *vs* 46.8%, $P = 0.006$). Among 3 subjects with gastric cancer, 2 subjects were infected with *H. pylori*.

Status of gastric mucosa

According to the updated Sydney system, 114 subjects (45.3%) were grade 0 for atrophy in the antrum, 131 subjects (51.9%) had grade 1 and 7 subjects (2.7%) had grade 2. None had grade 3. In the corpus, 220 cases (87.3%) were grade 0 for atrophy in the corpus and 27 and 5 cases (10.7% and 1.9%, respectively) were of grades 1, and 2 for atrophy, respectively. Therefore, 138 subjects (54.7%) had gastric mucosal atrophy in the antrum, and 32 (12.6%) subjects had gastric mucosal atrophy in the corpus when samples of grade 1 or more were considered atrophy-positive. The OLGA system was also used to assess the staging of gastritis; 109 (43.2%) was stages 0 and stage I was found in 52.3% (132/252). Stage Ⅱ was found in 3.9% (10/252). Stage Ⅲ was found only 1 (0.3%) subject and Stage Ⅳ were not found. The differences of histological scores according to the status of *H. pylori* infection were shown in Table 2. The scores for activity, inflammation, and atrophy both in antrum and corpus were significantly higher in *H. pylori*-positive subjects than negative subjects (all $P < 0.0001$). The score for intestinal metaplasia in the antrum was significantly higher in *H. pylori*-positive subjects than negative subjects ($P = 0.02$). Intestinal metaplasia in the antrum was found in 11.5% (14/121) in *H. pylori*-positive and 3.8% (5/131) in -negative subjects; therefore, the prevalence of intestinal metaplasia in the antrum was significantly higher in *H. pylori*-positive subjects than that of negative subjects ($P = 0.01$). OLGA score was also significantly higher in *H. pylori*-positive subjects than negative subjects (0.84 ± 0.56 *vs* 0.40 ± 0.52, $P < 0.0001$).

To evaluate predictive factors for the presence of atrophy, we performed a multivariate analysis. *H. pylori* infection was an independent risk factor for the presence of atrophy even after adjustment by age and gender ($P < 0.0001$, OR = 5.27, 95%CI: 3.02-9.18).

Gastric mucosal atrophy and PG in Myanmar

PG Ⅱ was significantly higher in *H. pylori*-positive than -negative subjects ($P < 0.001$); whereas there was no

Table 2 Differences of histological scores according to the status of *Helicobacter pylori* infection

	H. pylori (+)	*H. pylori* (-)	*P* value
n	121	131	
Age	42.5 ± 13.1	44.7 ± 15.2	0.22
Male	50	47	0.37
PG Ⅰ	86.9 ± 72.0	75.8 ± 77.0	0.006
PG Ⅱ	17.3 ± 11.6	9.8 ± 10.4	< 0.001
PG Ⅰ / Ⅱ	5.3 ± 2.0	8.1 ± 2.6	< 0.001
PG-positive	8	4	0.18
Antrum			
Activity	1.22 ± 0.82	0.08 ± 0.26	< 0.0001
Inflammation	1.53 ± 0.65	0.50 ± 0.54	< 0.0001
Atrophy	0.78 ± 0.52	0.39 ± 0.50	< 0.0001
Intestinal metaplasia	0.19 ± 0.59	0.05 ± 0.31	0.02
Corpus			
Activity	0.74 ± 0.65	0.08 ± 0.29	< 0.0001
Inflammation	0.99 ± 0.63	0.15 ± 0.42	< 0.0001
Atrophy	0.25 ± 0.50	0.05 ± 0.25	< 0.0001
Intestinal metaplasia	0.04 ± 0.32	0.04 ± 0.28	0.72
OLGA score	0.84 ± 0.56	0.40 ± 0.52	< 0.0001

OLGA: Operative link on gastritis assessment; *H. pylori*: *Helicobacter pylori*; PG: Pepsinogen.

Table 3 Levels of PG Ⅰ, PG Ⅱ, and PG Ⅰ/Ⅱ in atrophic gastritis (mean ± SD)

	Grade	*n*	PG Ⅰ	PG Ⅱ	PG Ⅰ/Ⅱ
Antrum	0	114	76.2 ± 82.0	10.2 ± 10.2	7.7 ± 2.4
	1	131	85.1 ± 68.9	15.7 ± 12.0	6.0 ± 2.7
	2	7	87.7 ± 57.4	20.9 ± 14.6	4.9 ± 2.2
	3	0	NA	NA	NA
Corpus	0	220	82.3 ± 77.1	12.6 ± 11.4	7.1 ± 2.5
	1	27	65.4 ± 40.5	17.6 ± 9.6	4.0 ± 2.2
	2	5	114.0 ± 105.1	23.9 ± 22.0	4.5 ± 2.4
	3	0	NA	NA	NA
OLGA	0	109	77.2 ± 83.6	10.1 ± 10.3	7.8 ± 2.3
	Ⅰ	132	82.6 ± 66.3	15.3 ± 11.2	6.0 ± 2.7
	Ⅱ	10	104.8 ± 84.1	24.8 ± 17.7	4.1 ± 1.8
	Ⅲ	1	67.8	8.7	7.8
	Ⅳ	0	NA	NA	NA

OLGA: Operative link on gastritis assessment; PG: Pepsinogen.

difference of PG Ⅰ among two group (Table 2). On the other hand, PG Ⅰ / Ⅱ was significantly lower in *H. pylori*-positive than -negative subjects ($P < 0.001$). When PG-positive was defined as the cutoff of PG Ⅰ levels ≤ 70 ng/mL and a PG Ⅰ / Ⅱ ratio ≤ 3.0, the percentage of PG-positive was higher in *H. pylori*-positive subjects [6.6% (8/121)] than that of *H. pylori*-negative subjects [3.0% (4/131)] although it did not reach the statistical significance ($P = 0.18$).

The overall prevalence of the PG-positive was only 4.7% (12/252). PG-positive was also significantly correlated with the presence of atrophy ($P = 0.012$). Among the 12 PG-positive subjects, 11 (91.6%) had atrophy. On the other hand, 132 (55.0%) out of 240 PG-negative subjects showed the presence of atrophy. Therefore, it means that when PG has high positive predictive value for the presence of atrophy; however it show high false-negative rate. Next, we examined the correlations between the severity of gastric mucosal atrophy and PGs (Table 3). In case of the antrum, PG Ⅰ and PG Ⅱ were significantly correlated with the severity of atrophy ($r = 0.13$, $P = 0.03$ for PG Ⅰ, $r = 0.34$, $P < 0.001$ for PG Ⅱ). On the other hand, PG Ⅰ / Ⅱ was significantly inversely correlated with the severity of atrophy ($r = -0.34$, $P < 0.001$). In case of the corpus, there was no correlation between PG Ⅰ and the severity of atrophy. PG Ⅱ were also significantly correlated with the severity of atrophy in the corpus ($r = 0.22$, $P < 0.001$). PG Ⅰ / Ⅱ was also significantly inversely correlated with the severity of atrophy in the corpus ($r = -0.37$, $P < 0.001$). The correlation between OLGA score and the severity of atrophy was also examined. PG Ⅱ were significantly correlated with the OLGA score ($r = 0.35$, $P < 0.001$). PG Ⅰ / Ⅱ was significantly inversely correlated with OLGA score ($r = -0.39$, $P < 0.001$). There was no correlation between PG Ⅰ and the OLGA score.

When we used the cut-off value of PG Ⅰ / Ⅱ as ≤ 3.0 for more than stage Ⅰ in the OLGA score, sensitivity and specificity were 8.3%, 99.0%, respectively. In case more than stage Ⅱ in the OLGA score, they were 18.1% and 95.4%, respectively. Therefore, we calculated the best cut-off value of PG Ⅰ / Ⅱ from ROC curve. For more than stage Ⅰ in OLGA score, the best cut-off value of PG Ⅰ / Ⅱ was 6.25 (sensitivity 62.9%, specificity 76.1%) [area under the ROC was 0.720 (95%CI: 0.657-0.782)]. For more than stage Ⅱ in OLGA score, the best cut-off value of PG Ⅰ / Ⅱ was 5.35 (sensitivity 81.8%, specificity 67.2%) [area under the ROC was 0.750 (95% CI: 0.610-0.889)].

DISCUSSION

We revealed that the prevalence of *H. pylori* in patients with dyspeptic symptoms in Myanmar was 48.0% by different 5 tests. In contrast to developed countries, *H. pylori* infections occur earlier in life and with a higher frequency in the developing world[18]. For example, the prevalence of *H. pylori* infection was decreasing according to the improvement of sanitary condition[19]. The present study showed that high prevalence of *H. pylori* infection was detected even in younger age group (41.9% at ≤ 29 years old) in Myanmar. Sanitary conditions such as a full equipment rate of water and sewage are considered as important factor for *H. pylori* infection[18]. The percentage of improved sanitation facilities in 2011 was still 77% in Myanmar (UNICEF, http://www.unicef.org/), which might be the reason for constant infection rate in every age group. The improvement of sanitary condition might be decreased *H. pylori* infection rate in Myanmar in the future. In addition, we found that higher prevalence of *H. pylori* infection was found in the largest city, Yangon compared with the second largest city, Mandalay. The percentage of usage of pit latrine is higher in Mandalay than in Yangon (Myanmar Multiple Indicator Cluster Survey 2009-2010, UNICEF, http://www.unicef.org/myanmar). In addition, drinking water

sources is more improved in Yangon than in Mandalay (http://www.unicef.org/myanmar). Therefore, it is difficult to explain the difference of *H. pylori* infection rate by the differences of sanitary condition. Unidentified genetic or host factors may result in them being less susceptible to *H. pylori* infection[20].

We found that 54.7% had mucosal atrophy in the antrum, and 12.6% subjects also had gastric mucosal atrophy in the corpus when samples of grade 1 or more were considered atrophy-positive. We previously reported that gastric mucosal atrophy was found in 91.9% in the antrum and 37.7% in the corpus in Bhutan where the incidence of gastric cancer is high (17.2 cases per 100000 population per year)[21]. Our study showed that another staging of gastritis (OLGA system) showed that most of case was stage 0-Ⅱ in Myanmar. Only one subject showed stage Ⅲ and none had stage Ⅳ. On the other hand, stage Ⅲ and Ⅳ were found in approximately 40% in Japan where the incidence of gastric cancer is quite high[22]. Furthermore, although it was significantly higher in *H. pylori*-positive than that of -negative subjects, the score of intestinal metaplasia in the antrum was lower in Myanmar than that of Japan (0.19 ± 0.59 in Myanmar, 0.50 ± 0.07 in Japan)[23]. Milder gastritis might be related with a moderate incidence of gastric cancer in Myanmar in spite of high *H. pylori* infection rate.

In this study, when PG-positive was defined as the cutoff of PG I levels ≤ 70 ng/mL and a PG Ⅰ/Ⅱ ratio ≤ 3.0, 55.0% of PG-negative subjects showed the presence of atrophy in Myanmar. Therefore, PG show high false-negative rate in Myanmar. The serum PG level can be affected by the ethnic background. In fact, the prevalence of low PG levels was the highest in the Indian compared to the Chinese and Malay populations even after adjustment for gender and *H. pylori* prevalence[24]. This showed that the serum PG criterion cannot be used in the Indian population for gastric cancer screening[25]. Other factors, such as age, gender, height, body weight, body surface area, smoking, and drinking habits, might be related to PG I and PG Ⅱ levels[26]. Therefore, different cutoff values used in different studies might affect the sensitivity and specificity of the results[27,28]. For example, in the Chinese population, the cutoff values for PG I and the PG Ⅰ/Ⅱ ratio used for the effective detection of atrophic gastritis were 82.3 ng/mL and 6.05, respectively[29]. In our study, we could not find any significant correlation between PG I and gastric mucosal atrophy in the corpus. On the other hand, PG Ⅰ/Ⅱ was significantly inversely correlated with the severity of atrophy both in the antrum and corpus. We found that the best cutoff value of PG Ⅰ/Ⅱ for more than stage I in OLGA score was 6.25 (sensitivity 62.9%, specificity 76.1%), and 5.35 (sensitivity 81.8%, specificity 67.2%) for more than stage Ⅱ in OLGA score. Future studies are needed to define the optimal PG cutoff values for gastric cancer screening in Myanmar.

The difference of the incidence of gastric cancer between China, Myanmar, India, and Thailand might be explain the difference of virulence factors of *H. pylori* in addition to the host factor and diet. Indeed, virulence factors of *H. pylori* have been revealed to be the predictors of gastric atrophy, intestinal metaplasia and severe clinical outcomes[4]. For example, CagA is the most studied virulence factor of *H. pylori*[4]. Western-type CagA is predominant in India, on the other hand, East-Asian-type CagA is predominant in China. It has been reported that East-Asian-type CagA strains are more virulent than Western-type CagA[4]. VacA is the second most extensively studied *H. pylori* virulence factor[30]. *vacA* s1 or m1 *H. pylori* strains have an increased risk of peptic ulcer or gastric cancer compared with those with s2 or m2 strains[30]. The prevalence of *vacA* m1 genotype was 73% in Thailand and approximately 60% in India[5,7,31]. Interestingly, recent study revealed that although CagA was translocated into a host cell, it did not persist for a long period by autophagy in response to *vacA* m1 but not m2[32]. On the other hand, the CagA expression was persisted in the CD44v9-expressing human gastric cancer cells[32]. A study to investigate virulence factors of *H. pylori* strains in Myanmar is now in progress. The genetic diversity of *H. pylori* strains in addition to environmental and host factors might be associated with the difference of the incidence of gastric cancer in Myanmar.

Another important finding was that the prevalence of *H. pylori* in patients with peptic ulcer was significantly higher than that of gastritis which consistent with previous reports[33-35]. This suggests that *H. pylori* infection can be a risk factor for the development of peptic ulcer even in Myanmar. Furthermore, histological scores were higher in *H. pylori*-positive subjects than negative subjects consistent with other report[23]. Therefore, eradication therapy for *H. pylori* infection can be contributed to the decreasing peptic ulcer in Myanmar.

However, our study includes several limitations. We obtained the samples from the patients living in Yangon and Mandalay which are the largest and the second largest cities in Myanmar. In general, the prevalence of *H. pylori* infection is higher in country sides than that of cities due to the difference of environmental factors including sanitary condition[18]. Therefore, our results cannot be generalized in Myanmar. In addition, we included only the patients with dyspeptic symptoms but not general population. The percentage of female was also higher than that of male although there was no difference of *H. pylori* infection rate between male and female. In general, the dyspeptic symptom is more common in female than in male[36]. In addition, we used the ELISA kit manufactured by Eiken Company in Japan for serology. It based on a Japanese *H. pylori* strain for the detection of *H. pylori* infection[37,38]. *H. pylori* antibody titers varied greatly depending on the test kit used[13,39]. It might be preferable to develop a domestic ELISA kit using *H. pylori* strains obtained in Myanmar for future studies.

In conclusion, the prevalence of *H. pylori* infection

in patients with dyspeptic symptoms in Myanmar was high in spite of moderate incidence of gastric cancer. On the other hand, most cases had mild gastritis. Strains isolated from Myanmar might be less virulent than those of East-Asian countries, but more virulent than those of India and Thailand. Furthermore, the presence of *H. pylori* was related with peptic ulcer and gastritis. Therefore, eradication therapy of *H. pylori* can contribute to decrease *H. pylori*-related diseases such as peptic ulcer and gastric cancer.

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COMMENTS

Background

The age-standardized incidence rate of gastric cancer in Myanmar was reported to be 11.2/100000 per year, which is higher than that of India and Thailand, and lower than that of China (6.1, 3.1 and 22.7/100000, respectively). Although the *Helicobacter pylori* (*H. pylori*) infection is the most important factor for the development of gastric cancer, the prevalence of *H. pylori* infection in Myanmar have not been elucidated.

Research frontiers

To understand the reason for higher incidence of gastric cancer in Myanmar than India or Thailand, it is important to elucidate of *H. pylori* infection rate in Myanmar. Furthermore, the gastric cancer risk can be assessed by the status of gastric atrophy. Not only endoscopic and histological examination but also the measurements of serum pepsinogen (PG) Ⅰ and PG Ⅰ / Ⅱ levels can be available to examine the status of gastric mucosal atrophy. However, the proper cut-off value can be various according to the geographic difference.

Innovations and breakthroughs

The prevalence of *H. pylori* infection in patients with dyspeptic symptoms in Myanmar was high in spite of moderate incidence of gastric cancer. On the other hand, most cases had mild gastritis. Strains isolated from Myanmar might be less virulent than those of East-Asian countries, but more virulent than those of India and Thailand. Furthermore, the presence of *H. pylori* was related with peptic ulcer and gastritis.

Applications

Eradication therapy of *H. pylori* can contribute to decrease *H. pylori*-related diseases such as peptic ulcer and gastric cancer.

Peer review

In this manuscript the authors evaluate the relation between *H. pylori* infection and atrophic gastritis in a Myanmar population. In agreement to literature data the study found a significant relation between two variables. The paper appears of clinical interest because these results are not previously reported in these geographic area.

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ORIGINAL ARTICLE

Observational Study

Performance of American Society for Gastrointestinal Endoscopy guidelines for dyspepsia in Saudi population: Prospective observational study

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Abstract

AIM: To evaluate adherence of primary care physicians (PCPs) to international guidelines when referring patients for upper-gastrointestinal endoscopy (UGE), evaluate the importance of alarm symptoms and the performance of the American Society for Gastrointestinal Endoscopy (ASGE) guidelines in a Saudi population.

METHODS: A prospective, observational cross-sectional study on dyspeptic patients undergoing UGE who were referred by PCPs over a 4 mo period. Refer-rals were classified as appropriate or inappropriate according to adherence to ASGE guidelines.

RESULTS: Total of 221 dyspeptic patients was enrolled; 161 patients met our inclusion criteria. Mean age was 40.3 years (SD ± 18.1). Females comprised 70.1%. Alarm symptoms included low hemoglobin level (39%), weight loss (18%), vomiting (16%), loss of appetite (16%), difficulty swallowing (3%), and gastrointestinal bleeding (3%). Abnormal endoscopy findings included gastritis (52%), duodenitis (10%), hiatus hernia (7.8%), features suggestive of celiac disease (6.5%), ulcers (3.9%), malignancy (2.6%) and gastroesophageal reflux disease (GERD: 17%). Among patients who underwent UGE, 63% met ASGE guidelines, and 50% had abnormal endoscopic findings. Endoscopy was not indicated in remaining 37% of patients. Among the latter group, endoscopy was normal in 54% of patients. There was no difference in proportion of abnormal endoscopic findings between two groups (P = 0.639).

CONCLUSION: Dyspeptic patients had a low prevalence of important endoscopic lesions, and none of the alarm symptoms could significantly predict abnormal

endoscopic findings.

Key words: Dyspepsia; Primary care physician; American Society for Gastrointestinal Endoscopy guideline; Upper gastrointestinal endoscopy; Saudi population

Core tip: A prospective study looking at the practice of primary care physicians in referring dyspeptic patients for endoscopy in Saudi Arabia, such study is the first prospective study to evaluate such practice in high *Helicobacter pylori* endemic area and the adherence of general practitioners to the international guidelines for a common gastroenterology disorder, and this will shed light on the approach for such disease.

Azzam NA, Almadi MA, Alamar HH, Almalki LA, Alrashedi RN, Alghamdi RS, Al-hamoudi W. Performance of American Society for Gastrointestinal Endoscopy guidelines for dyspepsia in Saudi population: Prospective observational study. *World J Gastroenterol* 2015; 21(2): 637-643 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/637.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.637

INTRODUCTION

Dyspepsia is a complex condition comprising a spectrum of chronic and recurrent symptoms related to the upper gastrointestinal tract. The cardinal symptoms are epigastric pain, discomfort, including postprandial fullness and early satiety, which may overlap with heartburn and regurgitation[1]. These symptoms could be the result of underlying organic pathology, such as chronic peptic ulcer disease, gastro-esophageal reflux or malignancy (organic dyspepsia). Dyspepsia can also present without evidence of organic cause (functional dyspepsia). Dyspepsia is a common condition that affects up to 80% of the population at some point during an individual's lifetime[2]. Multiple studies have shown that the condition is experienced by approximately 20%-40% of the general adult population and accounts for 3%-4% of all consultations in primary care[3-5]. In Western countries, studies have suggested that dyspepsia affects approximately a one-fourth of the population[6,7]. In Japan, India, and Turkey, the prevalence of dyspepsia has been estimated to be 17%, 30.4%, and 28.4%, respectively[8-10].

Dyspepsia is the most common indication for upper gastrointestinal endoscopy (UGE)[11-13]. It has been estimated that approximately 50% of all UGE referrals are dyspepsia related[14]. In approximately half of all dyspeptic cases, the endoscopic investigation reveals no underlying organic lesion[15-17].

The overwhelming number of dyspeptic patients referred for UGE has led to prolonged waiting times for endoscopic procedures, especially in the setting of open-access endoscopy units that allow general practitioners to request an endoscopic procedure without referral to a specialist. Concerns include the high cost, unnecessary burden on available resources and long waiting lists[18]. To reduce these problems and increase the effectiveness of endoscopy, adherence to treatment guidelines has been recommended[19,20]. Despite variability in composition, the recommendations of the majority of the guidelines are similar[21]. All suggest that dyspeptic patients who are over the age of 50 years and/or those with alarm symptoms at any age need urgent referral for endoscopy as an initial management strategy because endoscopy would change the management of this subset of patients[22-24]. In young patients without alarm symptoms, however, either a "test or treat" for *Helicobacter pylori* (*H. pylori*) in high-prevalence areas or an empirical acid-suppression trial are the initial management strategies of choice[24]. Saudi Arabia is considered to be a high prevalence area and estimated to be around 50%[25].

At King Khalid University Hospital (KKUH), the endoscopy unit is an open-access unit that receives a large number of referrals from various general and specialty clinics and from in-patient wards. Annually, more than eight thousand procedures are completed in the unit. The most common indication for UGE is dyspepsia, accounting for thousands of referrals, with approximately 50% from the primary care clinics[26]. This creates a significant burden on the allocated resources and negatively impacts waiting times. In this prospective study, we aimed to evaluate the adherence of primary care physicians (PCPs) to dyspepsia guidelines, to describe the common endoscopic findings, to evaluate the importance of "red flag" symptoms and to estimate the prevalence of *H. pylori* in dyspeptic patients. To our knowledge, this is the first study to evaluate such practices in an *H. pylori* high-prevalence region.

MATERIALS AND METHODS

Prospective, cross-sectional study on dyspeptic patients undergoing UGE in an open-access endoscopy unit was conducted. Data on all adult patients referred from PCPs to the Endoscopy Unit at KKUH, Riyadh, KSA, were prospectively collected over a period of 4 mo, starting from December 2012 and ending in April 2013. Dyspepsia was defined as chronic and recurrent epigastric pain or discomfort (including postprandial fullness and early satiety) with or without heartburn and regurgitation. Patients who had gastroesophageal reflux disease (GERD) -predominant symptoms such as heartburn or acid regurgitation alone, inflammatory bowel disease, a previously diagnosed malignancy or advanced liver disease were excluded from the study.

Upon presentation to the endoscopy unit, all patients who met our inclusion criteria were enrolled in the study and provided informed consent. The participants were interviewed by an endoscopist using a pre-designed data collection sheet (Table 1).

Table 1 Study variables

Variable	Description
Age	< 50 yr of age
	⩾ 50 yr of age
Gender	Male or female
Alarm symptoms	Anemia
	Hemoglobin level
	Male: < 13 g/dL
	Female: < 12 g/dL
	Weight loss of more than 4 kg
	Vomiting
	Loss of appetite
	Dysphagia
	Gastrointestinal bleeding
	Palpable abdominal mass
Other independent variables	Smoking
	Use of NSAID
	History of *Helicobacter pylori* treatment

NSAID: Non-steroidal anti-inflammatory medications.

Endoscopic findings were noted, and gastric biopsies were obtained to rule out *H. pylori* by utilizing the rapid urease test (Lencomm trade international, Poland). The biopsy samples were inoculated immediately into the rapid urease test gel. If the gel color changed within 20 min up to a maximum of 60 min the sample was considered positive for *H. pylori*.

Referrals were classified as appropriate or inappropriate according to adherence to ASGE guidelines. These included patients over the age of 50 years or those that presented with alarm symptoms at any age. Alarm symptoms included anemia, vomiting, loss of appetite, weight loss, gastrointestinal bleeding, dysphagia or the presence of a palpable abdominal mass. The endoscopic findings were categorized as normal or abnormal. Abnormal findings included gastritis, duodenitis, peptic ulcer, varices, features of celiac disease, hiatus hernia malignancy and others. Endoscopic findings were defined as important if the abnormalities included gastric or duodenal ulcers, varices, duodenitis, adenomatous polyps or malignancy[3].

Statistical analysis

Sample size calculation: Based on an *a priori* baseline prevalence of abnormal findings on endoscopy of 60%[25], Using the rule of 10 outcome events per predictor variable, and given we wished to include up to 9 variables in our multivariable model, we estimated that 150 individuals would be needed to provide sufficient accuracy within the multivariable analysis.

Data analysis: included descriptive statistics computed for continuous variables, including means, SD, minimum and maximum values, as well as 95%CI. Frequencies are used for categorical variables. We used hypothesis testing, the *t* test with unequal variances, as well as Fisher's exact test where appropriate.

Univariable and multivariable logistic regressions were used to examine the association between independent variables and the dependent variable the presence of an abnormality at endoscopy. Independent variables included; age, gender, smoking status, the use of non-steroidal anti-inflammatory medications (NSAIDs), history of weight loss, vomiting, loss of appetite, dysphagia, gastrointestinal bleeding, history of prior endoscopy, as well as the patients hemoglobin level as well as if they were infected with *H. pylori*. OR and 95%CI were calculated. Characteristics of test procedure (sensitivity, specificity) were used to evaluate the performance of the latest ASGE guidelines in detecting abnormalities on endoscopy.

We used the software STATA 11.2 (Stata Corp, TX, United States) in our analysis. A statistical significance threshold of $P = 0.05$ was adopted. No attempt at imputation was made for missing data.

RESULTS

A total of 221 patients were screened and 161 patients met our inclusion criteria. The mean age was 40.3 years (SD ± 18.1), and age ranged from 18 years to 98 years. Females represented 70.1% of the patients, while males represented 29.9%. The proportion of patients with alarm symptoms in our study was 39%; 39% had a low hemoglobin level, 18% had weight loss, 16% had vomiting, 16% had loss of appetite, 3% had difficulty in swallowing, 3% had gastrointestinal bleeding, and 2% had an epigastric mass on physical examination (Table 2). At least one alarm feature was observed in 79.4% of the females, and one alarm feature was observed in only 20.6% of the males (*P* value < 0.01). A proportion of the patients included in the study had incurred prior endoscopic procedures (29%); 60% of those had one prior endoscopy, 20% had two prior endoscopies, 6% had 3 prior endoscopies, and 12% had 4 previous endoscopies.

The mean hemoglobin level was 12.89 ± 0.17 g/dL.

According to the ASGE guidelines, 63% of the endoscopies were considered to be indicated; the results were abnormal in 50%, while 50% were normal.

Although 37% of the endoscopies were considered inappropriate, 54% had abnormal findings. There was no difference in the proportion of abnormal endoscopic findings between the two groups ($P = 0.639$; Table 3).

The most common endoscopic findings were gastritis in 52%, duodenitis in 10%, hiatus hernia in 7.8%, ulcers in 3.9% and malignancy in 2.6% of the patients; the remaining 17% were found to have reflux esophagitis signifying GERD. Furthermore, 6.5% had endoscopic features suggestive of celiac disease (Figure 1).

The rapid urease test was positive in 22% of the patients. The majority (62%) of those was younger than 50 years of age, and 20% had a history of receiving eradication therapy for *H. pylori*.

All procedures were completed successfully, and no adverse events occurred.

Table 2 Clinical characteristics of patients stratified by presence and absence of the normal and abnormal endoscopic finding as well as univariable analysis of all corresponding variables

Characteristics	Percentage of patients	Normal	Abnormal	*P* value	Univariable analysis OR (95%CI)
Female	70.1%	53%	47%	0.399	0.7 (0.37-1.47)
Male	29.9%	45%	55%	0.711	1.02 (0.97-1.56)
Age ⩾ 50	29%	60%	40%	0.094	1.01 (0.99-1.04)
Smoker	12%	42%	58%	0.671	1.49 (0.56-3.94)
Taking NSAID	14%	35%	65%	0.094	2.16 (0.86-5.44)
Vomiting	18%	47%	53%	0.285	1.25 (0.54-2.91)
Prior endoscopy	29%	38%	62%	0.039	2.06 (1.02-4.13)
Weight loss	16%	46%	54%	0.283	1.2 5(0.54-2.91)
Loss of appetite	16%	46%	54%	0.283	1.25 (0.54-2.91)
Dysphagia	3%	20%	80%	0.161	4.32 (0.47-39.52)
GI bleeding	3%	20%	80%	0.161	4.32 (0.47-39.52)
Epigastric mass	2%	0%	100%	0.075	
Low Hb	39%	41%	59%	0.245	1.08 (0.94-1.25)
Presence of *H. pylori*	22%	36%	64%	0.044	2.2 (1.01-4.87)

H. pylori: *Heliobacter pylori*; GI: Gastrointestinal; NSAID: Non-steroidal anti-inflammatory medications.

Table 3 Findings of endoscopy according to American Society for Gastrointestinal Endoscopy guidelines

Endoscopy finding	ASGE indicated (63%)	ASGE not indicated (37%)	*P* value
Normal	50%	54%	
Abnormal	50%	46%	0.6390
Important endoscopic finding	8%	3%	0.7806

ASGE: American Society for Gastrointestinal Endoscopy.

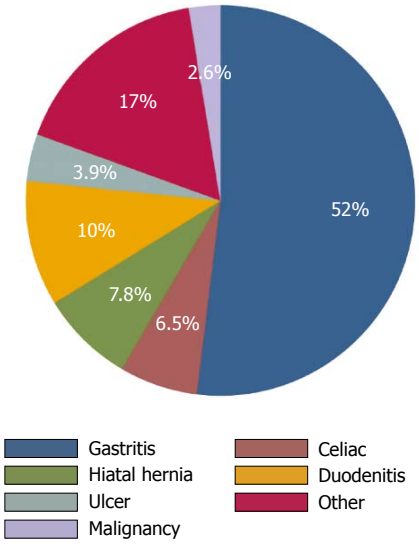

Figure 1 The distribution of abnormal endoscopic finding for the cohort.

Univariable and multivariable analysis

The only factors associated with the presence abnormal endoscopy on univariable analysis were *H. pylori* OR = 2.2 (95%CI: 1.01-4.87) and having undergone a previous endoscopy OR = 2.0 (95%CI: 1.02-4.13).

Using stepwise multivariable logistic regression, none of the variables included in the study could predict the finding of abnormalities at the time of endoscopy.

DISCUSSION

This prospective observational study found that overuse of upper gastrointestinal endoscopy is common in dyspeptic patients, between 25% and 40% of individuals with dyspepsia will consult a PCP as a result of their symptoms[27]. With such high prevalence, dyspepsia is a diagnostic and therapeutic challenge to physicians. Furthermore, most patients with dyspepsia have no detectable organic abnormality[22,28].Thus, endoscopic evaluation as an initial step in management is not recommended[9,29]. Endoscopic evaluation is recommended for older patients (older than 50 years), those with alarm symptoms, those taking NSAID, and those with persistent symptoms after acid suppression therapy and/or *H. pylori* eradication[18,30].

We found that approximately 40% of the patients complaining of upper abdominal symptoms had a normal finding according to the dyspepsia guidelines[20]. The specialty of the referring physicians affects the presumed etiology of upper gastrointestinal symptoms. The sensitivity and specificity for the diagnosis of functional dyspepsia was 61% and 84% for PCPs, respectively, while it was 73% and 37% for gastroenterologists, respectively[31]. In a large Canadian study, 1040 patients were evaluated for symptoms and underwent endoscopy within 10 d of referral. In this study, the predominant symptom was not predictive of the endoscopic findings, and the presence of alarm symptoms did not correlate with the demonstration of clinically significant endoscopic findings[16]. Another study evaluated alarm symptoms in functional dyspepsia and concluded that the value of symptoms in diagnosing functional dyspepsia was poor[32]. These data suggest that these symptoms are of limited value in the assessment of dyspepsia.

Our study confirmed that the majority of patients

with dyspepsia referred by PCPs had no important endoscopic lesion; approximately 40% were not indicated per the guidelines. Endoscopic abnormalities were found in only 48% of the patients; the majority had nonspecific gastritis, while important findings were observed in approximately 6%, with 2.6% of these patients having gastrointestinal malignancy. These results were similar to the findings of Choomsri *et al*[33] in which only important endoscopic lesions were found in 7% of the patients in the form of gastric ulcers and only 1% had gastric cancer. Moreover, in the present study, there were no clinical data such as age, smoking, NSAID use or alarm symptoms that could be used to predict the presence of important endoscopic lesions. This is in agreement with studies that found a poor positive predictive value for these symptoms[34,35]. It is thought that the presence of these alarm features are often indicative of advanced disease[36] and carry low diagnostic yield[37].

In young patients with uncomplicated dyspepsia, either a "test and treat" for *H. pylori* approach[38] or an empirical acid-suppression trial are recommended as first-line management strategies by most guidelines[20,39], depending on the prevalence of *H. pylori*.

The prevalence of *H. pylori* in the present study was 22%, and the majority of these patients were younger than 50 years (62%). Of interest, 20% of those testing positive for *H. pylori* had a previous history of receiving eradication therapy for *H. pylori*.

Abnormal endoscopic finding with important lesions were observed in only a small proportion of our study population, which is similar to a previous report[40]. The most cost-effective strategy in treating *H. pylori* is either empirical treatment or employment of a "test or treat" approach with consideration of endoscopy in a stepwise manner in dyspeptic patients, especially with the absence of alarm symptoms[41]. There is low prevalence of important endoscopic findings in *H. pylori* dyspeptic patients; therefore, a noninvasive method for diagnosing *H. pylori* would be the best modality rather than UGE because endoscopy remains a relatively expensive procedure and UGE is an invasive procedure that carries the risk of potential complications that may have grave consequences that exceed its benefit[42]. In our study, the presence of *H. pylori* was one of the predictors of an abnormal endoscopic finding. This has not been the case, however, according to multivariable analysis, which suggests the presence of an unmeasured confounder.

Gastroenterologists were found to be more likely than PCPs to comply with best practices for dyspepsia diagnosis and treatment, which could be due to PCPs having more concerns regarding long-term proton pump inhibitor use, which affects therapeutic decision making[26,43]. Studies showed an overall low dyspepsia guidelines compliance and such practice was observed in both developed and developing countries[25,33,40,43]. It is important to identify areas of disconnect between the guidelines and practices and to understand the predictors of low guideline compliance, which needs further studies employing larger populations.

The limitations of the present study included a relatively small sample size and the small number of important endoscopic lesions that were found, resulting in a low power to detect any clinically significant differences. Nonetheless, this study is one of the first prospective studies to address the appropriateness and diagnostic yield of endoscopy and adherence of PCPs to the international guidelines for dyspeptic patients in our region. We clearly demonstrate in this study the importance on adhering to the International dyspepsia guidelines when performing upper gastrointestinal endoscopy. We also believe that our hospital practices shed light on the medical approaches in our country that necessitate further studies. We, therefore advise the general practitioners to adopt these guidelines when evaluating patients with dyspepsia. Such practice would avoid unnecessary procedures and will result in an efficient utilization of resources.

In conclusion, the findings of the present study support selective UGE in patients with dyspepsia; a large number of UGE procedures in dyspeptic patients could be avoided. Further studies are needed to find prognostic markers for the abnormal findings in our patient population.

COMMENTS

Background

It's a prospective study looking at the practice of primary care physicians in referring dyspeptic patients for endoscopy in Saudi Arabia, such study is the first prospective study to evaluate such practice in high *Helicobacter pylori* endemic area and the adherence of general practitioners to the international guidelines for a common gastroenterology disorder, and this will shed light on the approach for such disease.

Research frontiers

To evaluate the adherence of primary care physicians to international guidelines when referring patients for upper-gastrointestinal endoscopy, describe the most common endoscopic findings, evaluate the importance of alarm symptoms and the performance of the American Society for Gastrointestinal Endoscopy (ASGE) guidelines in a Saudi population.

Innovations and breakthroughs

It's a first of its kind prospective study looking at the practice of primary care physicians in referring dyspeptic patients for endoscopy in Saudi Arabia, such study might change the current practice of referring system for dyspeptic patients for upper gastrointestinal (GI) endoscopy, especially those with no alarming symptoms.

Applications

This proposal might change the current practice of referring system for dyspeptic patients for upper GI endoscopy, especially those with no alarming symptoms.

Peer review

In this study, the authors have tried to demonstrate factors related to the positive endoscopic findings based on the ASGE guideline/ Study design is nice and analysis is clear. Their results should be useful for the general readers.

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ORIGINAL ARTICLE

Prospective Study

Profiling cellular bioenergetics, glutathione levels, and caspase activities in stomach biopsies of patients with upper gastrointestinal symptoms

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Abstract

AIM: To measure biochemical parameters in stomach biopsies and test their suitability as diagnostic biomarkers for gastritis and precancerous lesions.

METHODS: Biopsies were obtained from the stomachs of two groups of patients (n = 40) undergoing fiber-optic endoscopy due to upper gastrointestinal symptoms. In the first group (n = 17), only the corpus region was examined. Biopsies were processed for microscopic examination and measurement of mitochondrial O_2 consumption (cellular respiration), cellular adenosine triphosphate (ATP), glutathione (GSH), and caspase activity. In the second group of patients (n = 23), both corpus and antral regions were studied. Some biopsies were processed for microscopic examination, while the others were used for measurements of cellular respiration and GSH level.

RESULTS: Microscopic examinations of gastric corpus biopsies from 17 patients revealed normal mucosae in 8 patients, superficial gastritis in 7 patients, and chronic atrophic gastritis in 1 patient. In patients with normal histology, the rate (mean ± SD) of cellular respiration was 0.17 ± 0.02 μmol/L O_2 min^{-1} mg^{-1}, ATP content was 487 ± 493 pmol/mg, and GSH was 469 ± 98 pmol/mg. Caspase activity was detected in 3 out of 8 specimens. The values of ATP and caspase activity were highly variable. The presence of superficial gastritis had insignificant effects on the measured biomarkers. In the patient with atrophic gastritis, cellular respiration was high and

ATP was relatively low, suggesting uncoupling oxidative phosphorylation. In the second cohort of patients, the examined biopsies showed either normal or superficial gastritis. The rate of cellular respiration (O_2. μmol/L min^{-1} mg^{-1}) was slightly higher in the corpus than the antrum (0.18 ± 0.05 *vs* 0.15 ± 0.04, P = 0.019). The value of GSH was about the same in both tissues (310 ± 135 *vs* 322 ± 155, P = 0.692).

CONCLUSION: The corpus mucosa was metabolically more active than the antrum tissue. The data in this study will help in understanding the pathophysiology of gastric mucosa.

Key words: Stomach; Gastritis; Mitochondria; Gastric mucosa; Cellular respiration

Core tip: Using small gastric mucosal biopsies obtained from patients with upper gastrointestinal symptoms, several cellular bioenergetic and dynamic parameters were measured and correlated with the histopathological features of the gastric mucosa.

Alfazari AS, Al-Dabbagh B, Al-Dhaheri W, Taha MS, Chebli AA, Fontagnier EM, Koutoubi Z, Kochiyi J, Karam SM, Souid AK. Profiling cellular bioenergetics, glutathione levels, and caspase activities in stomach biopsies of patients with upper gastrointestinal symptoms. *World J Gastroenterol* 2015; 21(2): 644-652 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/644.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.644

INTRODUCTION

The gastric mucosa of the normal human stomach includes numerous tubular epithelial glands. In the corpus region, each gland is lined by a heterogeneous population of cells secreting mucus, acid, pepsinogen, and various hormones and peptides[1]. In the antrum, the glands produce mainly mucus, hormones, and peptides. Analysis of gastric mucosal tissues from patients undergoing endoscopic examination (for recurrent upper gastrointestinal symptoms) and comparing them with gastric cancer tissues obtained from three different regions (safe margin, tumor edge, and tumor center) revealed that these tissues represent the multistep process of gastric carcinogenesis[2]. The sequential changes in the morphology of the gastric glands coincide with increased proliferating stem/progenitor cells during progression from normal to gastritis, into metaplasia, and finally into adenocarcinoma. Indeed when a stem cell-specific marker (Oct4) was used, the labeling pattern and the measurement of Oct4 protein content supported the central role of stem cells in driving precancerous and cancerous changes[3]. Since proliferation of gastric stem/progenitor cells and alteration of cellular dynamics is an important event in carcinogenesis, measurement of the cellular bioenergetics of gastric mucosal biopsies would be an emerging need.

Cellular bioenergetics reflects the biochemical processes involved in the energy metabolism (energy conversion or transformation). Cellular respiration implies the delivery of O_2 and metabolic fuels to the mitochondria, the oxidation of reduced metabolic fuels with passage of electrons to O_2, and the synthesis of adenosine triphosphate (ATP)[4]. Impaired bioenergetics therefore entails disturbances in these processes.

Cellular mitochondrial O_2 consumption is a highly sensitive biomarker for detecting tissue derangements[5]. Impairments in cellular membranes, mitochondria, or metabolic enzymes are expected to disrupt energy kinetics within the cell. Cells with intact bioenergetics are more capable of repairing damage. Furthermore, apoptosis with activation of caspases is more likely to result in cell death if associated with impaired cellular bioenergetics[6]. Therefore, energy metabolism has a significant impact on the fate of the cell. This notion stems from the dependency of human biological systems on aerobic metabolism. Cancer cells, on the other hand, may survive on anaerobic metabolism, a phenomenon commonly referred to as aerobic glycolysis or the Warburg effect[7].

Several human and animal studies have demonstrated that bioenergetics of the gastric epithelium are affected by various diseases (*e.g.*, ischemia) and toxins (*e.g.*, acetylsalicylic acid and non-steroidal anti-inflammatory drugs)[8-11]. Similarly, gastric tissue deficient in superoxide dismutase (a parietal cell enzyme that prevents the accumulation of superoxides) has mitochondrial dysfunction and perturbed energy metabolism, which manifests *via* reduced ATP and increased apoptosis[9].

Cellular bioenergetics has been used as a biomarker for metabolic diseases[12]. In the present study, cells and tissues obtained from patients were used to diagnose impaired cellular bioenergetics. The main aim of the present study was to show the feasibility of performing the same measurements [cellular respiration, ATP, glutathione (GSH), and caspase activity] on small gastric mucosal biopsies. The results here demonstrate the feasibility of measuring cellular mitochondrial O_2 consumption, ATP, GSH, and apoptosis in small mucosal biopsies from the stomach of patients.

MATERIALS AND METHODS

Materials

Pd(Ⅱ) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Monobromobimane (mBBr, MW 271.111) was purchased from Molecular Probes (Eugene, Oregon). A lyophilized powder of caspase inhibitor I [*N*-benzyloxycarbonyl-Val-Ala-Asp (O-methyl)-fluoromethylketone; zVAD-fmk; MW 467.5; pan-caspase inhibitor] was purchased from Calbiochem (La Jolla, CA). Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; MW 675.64; caspase-3

Table 1 Gastric corpus histology and measured biomarkers in the first cohort of patients (n = 17)

Patients	Age (yr)	Gender	Medications	Clinical findings	*H. pylori*	Histology	k_c	ATP	GSH	AMC
1	48	F	Mebeverine	Hysterectomy	-	Normal	0.18	1547 ± 5.6	396	0
2	38	F	PPI Thyroxin	Peptic ulcer Thyroid cancer	ND	Normal	0.19	13 ± 0.4	404	0
3	52	F	PPI NSAID Progesterone	IBS Thyroid neoplasm	+	Normal	0.13	267 ± 0.3	517	0
4	20	F	-	Hiatal hernia	-	Normal	0.18	492 ± 5.0	570	0
5	50	M	PPI, Losartan, Prednisolone	Acromegaly Hypertension	-	Normal	0.16	336 ± 3.8	360	0
6	34	M	Morbid obesity	Morbid obesity	+	Normal	0.16	495 ± 7.0	638	9
7	23	M	PPI Aspirin	Hypertension Dyslipidemia	-	Normal	0.18	14 ± 1.2	461	36
8	52	F	PPI Calcium, vitamin D Atorvastatin	-	-	Normal	0.19	731 ± 5.2	403	63
mean ± SD	40 ± 13						0.17 ± 0.02	487 ± 493	469 ± 98	
9	38	F	antacid	Mesenteric cyst	-	Superficial gastritis	0.15	1525 ± 8.7	476	95
10	22	M	PPI	-	+	Superficial gastritis	0.20	90 ± 4.1	347	14
11	46	F	PPI	-	-	Superficial gastritis	0.18	949 ± 1.3	373	14
12	28	F	PPI	IBS Hyperthyroidism Depression	+	Superficial gastritis	0.18	11 ± 0.2	830	13
13	65	M	PPI	-	-	Superficial gastritis	0.21	14 ± 0.9	496	0
14	62	F	PPI	-	+	Superficial gastritis	0.14	56 ± 2.0	366	0
15	55	F	PPI Tamoxifen	Breast cancer	-	Superficial gastritis	0.17	ND	ND	ND
mean ± SD	47 ± 18						0.18 ± 0.04	370 ± 563	481 ± 182	
16	72	F	PPI	Diabetes mellitus Hypertension Dyslipidemia Breast cancer	+	Atrophic gastritis	0.27	275 ± 4.2	606	2
17	36	M	-	Morbid obesity	+	Inadequate	0.14	37 ± 0.1	1138	105

kc, in μmol/L O_2 min^{-1} mg^{-1}; GSH in pmol/mg; ATP in pmol/mg; AMC: Peak area in arbitrary unit/mg × 10^3. ND: Not detectable; IBS: Irritable bower syndrome; NASID: Non-steroidal anti-inflammatory drugs; PPI: Proton pump inhibitors; *H. pylori*: *Helicobacter pylori*.

substrate) was purchased from Axxora LLC (San Diego, CA). Recombinant human active caspase-3 was purchased from BD Pharmingen™ (Becton Dickinson & Company, Franklin Lakes, NJ, United States). Glucose, 5,5'-dithio-bis(2-nitrobenzoic acid) [DTNB, MW 396.35, molar extinction coefficient at 412 nm 13.6 × 10^3], GSH (MW 307.43; pK_a 8.7), HPLC-grade methanol, dichloromethane, trifluoroacetic acid (TFA), methanesulfonic acid (MSA), and remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

GSH was prepared in dH_2O and its concentration was measured by Ellman's reagent[9]. The GS-bimane derivative (GSH standard), sodium methane sulfonate (NaMS), mBBr, and DTNB solutions were prepared and stored as described[13-15]. DTNB working solution was 0.2 mmol/L DTNB in 100 mmol/L Tri-Cl (pH 8.0). GSH standard (2 μmol/L) was used to generate a calibration curve with each analytical run, which was linear from 10 to 200 picomoles ($R \geqslant 0.982$).

zVAD-fmk (2.14 mmol/L) and Ac-DEVD-AMC (7.4 mmol/L) were prepared in dimethyl sulfoxide and stored at -20 ℃. Pd phosphor (2.5 mg/mL = 2 mmol/L), sodium cyanide (CN, 1.0 mol/L), and glucose oxidase (10 mg/mL) were prepared in dH_2O and stored at -20 ℃.

Ethics

This work is compliant with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved by the Institutional Review Board for the protection of human subjects, Al Ain Medical District Human Research Ethics Committee (Protocol No. 12/49 CRD 199). All patients provided informed written consent.

Gastric biopsies

The first cohort involved 17 patients who were admitted to the Endoscopy Unit of Tawam Hospital (Al Ain City, Abu Dhabi) for diagnostic fiber-optic endoscopy due to recurrent upper gastrointestinal symptoms (dyspepsia, abdominal pain, and heartburn) (Table 1). After collecting samples for standard patient care, five to eight additional mucosal biopsies were collected for the purpose of this

Table 2 Measured biomarkers in gastric corpus *vs* antrum in the second cohort of patients (n = 23)

Patients	Age (yr)	GI presentation	Gender	Gastric corpus		Gastric antrum	
				k_c	GSH	k_c	GSH
18	27	Liver lesion	F	0.11	357	0.12	378
19	71	Hypertension, diabetes, dyslipidemia, dyspepsia	M	0.13	627	0.16	379
20	22	Dyspepsia	M	0.13	366	0.25	331
21	18	Dyspepsia	F	0.14	319	0.19	260
22	30	Dyspepsia	M	0.16	733	0.21	853
23	34	Thyroidectomy, dyspepsia	F	0.14	449	0.18	408
24	24	Familial Mediterranean fever, dyspepsia	M	0.17	256	0.14	243
25	66	Diabetes, gastritis	M	0.15	243	0.12	317
26	69	Prostate cancer, aortic aneurysm, dyspepsia	M	0.22	269	0.13	470
27	22	Morbid obesity, dyspepsia	M	0.15	307	0.13	256
28	30	Dyspepsia	M	0.15	351	0.17	347
29	46	Obesity, dyspepsia	F	0.22	256	0.14	356
30	21	Dyspepsia	F	0.15	260	0.12	342
31	49	Hypertension, diabetes, ovarian, and cervical cancers, dyspepsia	F	0.18	202	0.10	104
32	44	Hypertension, diabetes, dyslipidemia, dyspepsia	F	0.18	180	0.17	166
33	34	Dyspepsia	F	0.20	218	0.11	256
34	18	Thalassemia major, s/p BMT, dyspepsia	F	0.15	219	0.12	193
35	81	GERD, esophagitis, hiatal hernia	M	0.19	279	0.11	219
36	62	Dyspepsia	F	0.28	258	0.19	554
37	40	Morbid obesity, dyslipidemia, chronic renal failure, dyspepsia	F	0.13	301	0.11	326
38	26	Data not available	F	0.30	305	0.18	177
39	39	Data not available	M	0.23	214	0.13	260
40	32	Data not available	F	0.21	166	0.12	211
mean ± SD	40.4 ± 19.8			0.18 ± 0.05	310 ± 135	0.15 ± 0.04	322 ± 155

kc, in μmol/L O_2 min^{-1} mg^{-1}; GSH: Glutathione, in pmol/mg; s/p. BMT: Status post bone marrow transplantation; GERD: Gastroesophageal reflux disease; GI: Gastrointestinal.

study. The samples (7.7-30 mg) varied in dimensions from 1 mm × 1 mm to 2 mm × 3 mm. Tissue samples were processed for histological examination and measurements of cellular respiration, caspase activity, ATP, and GSH. Values of the measured biomarkers were expressed per specimen as wet weight (in mg). For consistency, studied samples were obtained from the gastric corpus (body) midway along the greater curvature. In a separate cohort of 23 patients, samples were obtained from the corpus and the antrum; these additional samples were processed for histology, cellular respiration, and GSH only (due to limited sample availability) (Table 2).

For histological examination, tissue samples were processed as previously described[2]. *Helicobacter pylori* (*H. pylori*) infection was detected using Warthin-Starry stain[16] or urease-based test (campylobacter-like organism test, Ptonto DryTM, Medical Instruments Corporation, Brignais, France).

Within 20 min of sample collection, the specimens were transferred to 1.0 mL RPMI containing 0.5% fat-free bovine albumin and 3 μmol/L Pd phosphor and processed for O_2 measurements at 37 ℃ as previously described[17-19].

For measuring cellular ATP, a specimen from each patient was immediately homogenized in 0.5 mL of ice-cold 2% trichloroacetic acid for 2 min. The supernatants were collected by centrifugation (1000 *g* at 4 ℃ for 5 min) and stored at -20 ℃ until analysis as previously described[17-19].

For GSH labeling with mBBr, the reaction solution containing the gastric specimen (7.7-30 mg) was incubated at 25 ℃ for 15 min. The reaction was stopped with 100 μL of 70% perchloric acid and diluted with 400 μL of 10 mmol/L Tris-MSA. The tissue was vortexed, homogenized, and centrifuged. The supernatant was stored at -20 ℃ until HPLC analysis[13-15].

For measuring caspase activity, two specimens from each patient were used. They were immediately placed in 1.0 mL RPMI containing 37 μmol/L Ac-DEVD-AMC with and without 32 μmol/L zVAD-fmk as previously described[17-19].

HPLC

The reversed-phase HPLC system (Waters, Milford, MA, United States) was used. Ultrasphere IP column, 4.6 mm × 250 mm (Beckman, Fullerton, CA, United States) was operated at 25 ℃ at 1.0 mL/min. For GSH determination, solvent A was 0.1% (v/v) trifluoroacetic acid/water and solvent B was HPLC-grade methanol. The flow rate was 1.0 mL/min. The employed gradient was: 0 min, 10% B; 5 min 10% B; 13 min, 100% B; 15 min, 10% B; 20 min, re-inject. The excitation and emission wavelengths were 390 nm and 480 nm, respectively. The injection volume was 50 μL.

For AMC detection, the excitation wavelength was 380 nm and the emission wavelength 460 nm. Solvents A and B was HPLC-grade methanol: dH_2O 1:1 (isocratic). The run time was 15 min and the injection volume was 50 μL.

Statistical analysis

Data were analyzed using SPSS statistical package (version 19). The nonparametric Mann-Whitney test (2 independent variables) was used to compare samples.

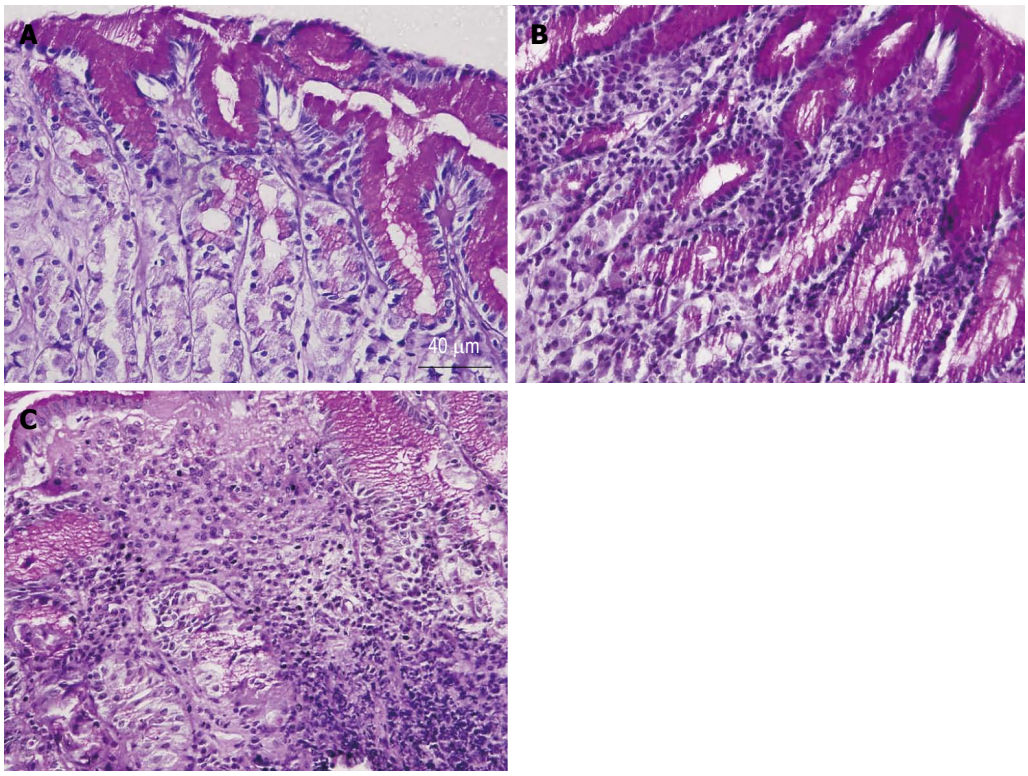

Figure 1 Representative micrographs of gastric corpus mucosal sections showing normal mucosa (A), superficial gastritis (B), and chronic atrophic gastritis (C). Note the mild infiltration of the gastric mucosa by lymphoid cells near the luminal surface in superficial gastritis (B) and the massive infiltration of the mucosa by lymphoid cells in atrophic gastritis (C).

RESULTS

Gastric corpus specimens were collected from the first 17 patients; their results are summarized in Table 1. The patients' age averaged 44 ± 16 years; 11 patients (65%) were females. All patients had recurrent upper gastrointestinal symptoms (dyspepsia, abdominal pain, and heartburn). Twelve (71%) patients were receiving proton pump inhibitors (PPI). Biopsies of 7 patients (41%) tested positive for *H. pylori* (Table 1). Microscopic examination of 5-micron-thick gastric mucosal sections revealed that 8 patients had normal gastric mucosa (Figure 1A). The biopsies of 7 patients had chronic superficial gastritis with infiltration of the luminal side of the mucosa with some inflammatory cells (Figure 1B). The gastric mucosa of only one patient (Patient 16) revealed evidence of chronic atrophic gastritis with massive infiltration with inflammatory cells (Figure 1C). The biopsy of one patient was inadequate for microscopic examination.

O_2 consumption by the stomach biopsy of Patient 16 (a patient with atrophic gastritis) is shown in Figure 2A. The rate of cellular respiration was the highest (0.27 μmol/L O_2 mg^{-1} min^{-1}), but cellular ATP was below the average (275 pmol/mg) (Table 1). This result suggested uncoupling oxidative phosphorylation (a state of high mitochondrial O_2 consumption with low cellular ATP) as a mechanism of the enhanced respiration. O_2 consumption was completely inhibited by cyanide, confirming that the oxidation occurred in the mitochondrial respiratory chain. The addition of glucose oxidase (which catalyzes the reaction of *D*-glucose + O_2 to *D*-glucono-δ-lactone + H_2O_2) depleted the remaining O_2 in the solution.

The rates of cellular mitochondrial O_2 consumption (k_c, μmol/L O_2 mg^{-1} min^{-1}) were 0.17 ± 0.02 for the 8 normal histology patients and 0.18 ± 0.03 for the 7 superficial gastritis patients (P = 0.867). The corresponding values for cellular ATP were 487 ± 493 and 370 ± 563, respectively (P = 0.573). The large variation in cellular ATP was likely due to sample processing. Nevertheless, the data show that superficial gastritis was not associated with bioenergetic changes in the gastric mucosa.

Representative GSH standard HPLC run and GSH standard curve are shown in Figure 2B; of note, GSH labeling with mBBr was blocked by N-ethylmaleimide (data not shown). Representative GSH run of acid-soluble supernatant of the stomach biopsy of Patient 10 (a patient with superficial gastritis) is shown in Figure 2C. Cellular GSH for the 8 patients with normal histology was 469 ± 98, and 481 ± 182 for the 7 patients with superficial gastritis (P = 0.662) (Table 1). Consistently, superficial gastritis was not associated with GSH changes in the gastric mucosa.

Ac-DEVD-AMC cleavage by the recombinant human active caspase-3 is shown in Figure 2D. The reaction, in 1.0 mL RPMI, contained 100 ng caspase-3 with and without 32 μmol/L zVAD-fmk (pan-caspase inhibitor). The mixtures were incubated at 37 ℃ for 10 min. Ac-DEVD-AMC (37 μmol/L) was then added and the incu-

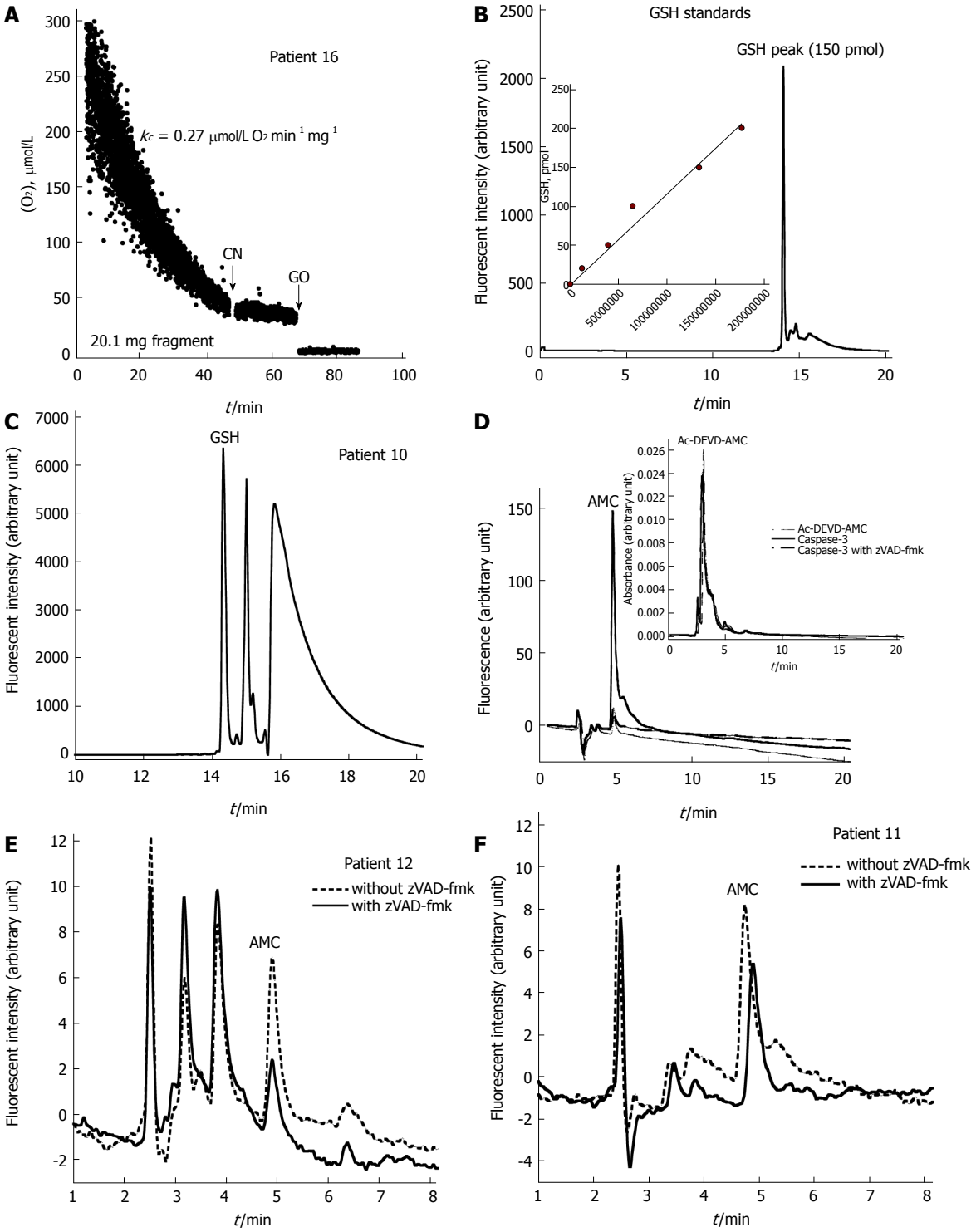

Figure 2 Representative measurements of gastric corpus cellular respiration, glutathione, and caspase activity. A: A run of cellular mitochondrial O_2 consumption by the gastric mucosa of Patient 16. The rate of respiration (k, μmo/L O_2 min^{-1}) was set as the negative of the slope of [O_2] *vs t*. The value of k_C (μmo/L O_2 min^{-1}) and the additions of 10 mmo/L cyanide (a specific inhibitor of cytochrome oxidase) and 50 μg/mL glucose oxidase (catalyzes the reaction of *D*-glucose + O_2 to *D*-glucono-δ-lactone + H_2O_2) are shown. O_2 consumption was inhibited by cyanide, confirming the oxidation occurred in the mitochondrial respiratory chain. O_2 was depleted by the addition of glucose oxidase, confirming the presence of dissolved O_2; B: A representative HPLC run of 150 pmol glutathione (GSH) standard (GSH retention time = 14.2 min); GSH standard curve is also shown [insert; GSH (pmol) = 0.00000117 x GSH peak area]; C: A representative HPLC run of cellular GSH in a stomach biopsy (23.9 mg mucosal fragment) from Patient 10. GSH peak area was 354508365 arbitrary units per 50 μL injection volume (reaction volume = 1.0 mL). Thus, cellular GSH content = 347 pmol mg^{-1} [(354508365 × 0.00000117 × 20)/23.9]; D: Representative HPLC runs for the Ac-DEVD-AMC cleavage reaction by human active caspase-3 with and without the pan-caspase inhibitor zVAD-fmk. The caspase-3 substrate Ac-DEVD-AMC was detected by absorbance at 380 nm with a retention time of about 3 min (insert). The product AMC was detected by fluorescence (380 nm excitation and 460 nm emission) with a retention time of about 4.8 min; E: Representative HPLC runs of caspase activities in the presence (solid line; 22.3 mg mucosal fragment) and absence (dashed line; 21.2 mg mucosal fragment) of the pan-caspase inhibitor zVAD-fmk for Patient 12. The AMC peak area without zVAD-fmk was 16015 arbitrary units/mg and with zVAD-fmk 2810 arbitrary units/mg. Intracellular caspase activity was set as AMC peak area without zVAD-fmk minus with zVAD-fmk, or 13205 (rounded down to 13 × 10^3) (Table 1); F: Representative HPLC runs of caspase activities with (solid line; 23.3 mg mucosal fragment) and without (dashed line; 19.1 mg mucosal fragment) zVAD-fmk for Patient 11. The AMC peak area without zVAD-fmk was 51207 arbitrary units/mg and with zVAD-fmk 37086 arbitrary unit mg^{-1}. Intracellular caspase activity, thus, was about 14 × 10^3 (Table 1).

bation continued at 37 ℃ for an additional 20 min. Ac DEVD-AMC was detected by absorbance at 380 nm with a retention time of approximately 3 min (Figure 2D). The product AMC was detected by fluorescence (380 nm excitation and 460 nm emission) with a retention time of about 5 min (Figure 2D). The cleavage reaction was inhibited by zVAD-fmk (Figure 2D). Caspase activity was set as the AMC peak area without zVAD-fmk minus the AMC peak area with zVAD-fmk. Representative HPLC runs of caspase-3 activity in the gastric corpus of Patients 12 and 11 are shown in Figure 2E and F, respectively. Caspase activity was detected in 3 of 8 (38%) patients with normal histology and 5 of 7 (71%) patients with abnormal histology (Table 1).

H. pylori had no significant effect on the rate of respiration, level of ATP, cellular GSH, or intracellular caspase activity ($P > 0.121$). Non-significant effects were also noted with respect to the use of PPI ($P > 0.104$).

The second cohort involved gastric corpus and antrum specimen collection from 23 additional patients. Due to limited sample availability, these biopsies were processed only for histology, cellular respiration, and GSH measurements (Table 2). Tissue samples for histology, however, were only available for 7 out of 23 (30%) patients; all had either normal or varying degrees of superficial (mild) gastritis. The patients' age averaged 40.4 ± 19.8 years; 14 patients (61%) were females. The rate of respiration (μmol/L O_2 mg^{-1} min^{-1}) was slightly higher in the corpus than the antrum (0.18 ± 0.05 *vs* 0.15 ± 0.04, $P = 0.019$). The value of GSH was about the same in both tissues (310 ± 135 *vs* 322 ± 155, $P = 0.692$).

DISCUSSION

Bioenergetic studies on the gastric epithelium are relatively limited, especially with respect to investigating human stomach diseases and the use of compound biomarkers[8,10,11,20-30]. The main purpose of this study was to examine the suitability of using biochemical parameters (cellular respiration, ATP, GSH, and caspase activity) as biomarkers for the gastric mucosa. The success of these measurements relies on the appropriate processing of the samples at the site of tissue collection. For O_2 measurements, the tissue should be immediately placed in ice-cold RPMI medium saturated with 95% O_2 and 5% CO_2. The sample should then be transferred to the laboratory on wet-ice and processed for the O_2 measurement within a few minutes of collection. For ATP, the tissue should be immediately quenched (at the procedure site) with acidic solution (freshly-made) to prevent ATP hydrolysis by cellular ATPases. For GSH, the tissue should be immediately immersed (at the procedure site) in thiol derivatization reaction that contains a large excess of mBBr (5 mmol/L). The GS-bimane derivatives are stable and can be stored until HPLC analysis. For caspase activity, the sample should be immediately placed in the Ac-DEVD-AMC cleavage reaction at the procedure site.

Having adhered to these experimental procedures, the values for the rate of cellular respiration (CV ≤ 17%) and GSH content (CV ≤ 48%) were reasonably consistent within the studied biopsies (Tables 1 and 2). These results were noted despite the wide-spectrum of clinical and histological variations among the patients and samples. Thus, cellular O_2 consumption and GSH are relatively preserved in the gastric mucosa. Cellular ATP (CV = 120%) and caspase activity (CV = 108%) were markedly varied however, likely due sample processing (Table 1).

We do identify that there are limitations to this study, as the sample size is relatively small and includes patients with minor gastric pathology. The clinical significance of these measurable biomarkers needs to be explored in future studies in patients with various pathologies, such as *H. pylori* infection, and the use of PPI.

Patient 3 had the lowest rate of respiration (0.13 μmol/L O_2 mg^{-1} min^{-1}); she had benign thyroid neoplasm and was taking multiple medications, including thyroxin, PPI, diclofenac, and medroxyprogesterone. Nevertheless, the cellular ATP, GSH, and caspase activity were not significantly different (Table 1).

Patient 16 had atrophic gastritis. Her rate of respiration was the highest (0.27 μmol/L O_2 mg^{-1} min^{-1}). She also had other complicated clinical problems (*e.g.*, diabetes mellitus, hypertension, dyslipidemia, and breast cancer) and was on PPI. While the rate of cellular respiration was the highest, the cellular ATP level was below average (275 pmol/mg) (Table 1), suggesting uncoupling oxidative phosphorylation.

Bioenergetics of the gastric epithelium was investigated in specimens collected from animal and human tissues[8,10,11,20]. In the bullfrog gastric mucosa, cellular mitochondrial O_2 consumption was increased and cellular ATP was decreased in the presence of acetylsalicylic acid[8]. Deficits in gastric cellular bioenergetics are also documented in shock and ischemia[10]. Non-steroidal anti-inflammatory drugs (NSAID) are shown to uncouple mitochondrial oxidative phosphorylation (lowering cellular ATP) in the gastric tissue[11]. Patient 6 was on aspirin and his cellular ATP was low (14 pmol/mg) (Table 1).

Activation of the mitochondrial apoptotic pathway is essential for *H. pylori*-induced apoptosis in gastric epithelial cells[21]. The *H. pylori* vacuolating cytotoxin A (vacA) causes direct mitochondrial disturbances and alterations in the bioenergetics of gastric epithelial cells[24]. Here, *H. pylori* had no noticeable effects on kc, ATP, GSH, or caspase activity. Nevertheless, the impact of *H. pylori* on the studied biomarkers requires a much larger sample size and appropriately selected control group.

Oxidative phosphorylation was measured in permeabilized corpus mucosal biopsies[22]. Cellular respiration was about 2-fold lower in patients with atrophic gastritis compared to non-atrophic gastritis. This effect was attributed to a deficiency of complex I of the respiratory chain[22]. Furthermore, limiting cellular bioenergetics was proposed to cause dysfunction of the zymogenic mucosal cells[23]. These studies demonstrate that stomach mucosal diseases can be associated with altered oxidative phosphorylation[23].

Activation of caspases permeabilizes (uncouples) the inner mitochondrial membrane, resulting in the collapse of the proton motive force, loss of electrochemical potential, and uncoupling of oxidative phosphorylation[25]. These processes lead to the rapid depletion of cellular nutrients, metabolic fuels, and ATP. The gastric mucosa is an intensely energy-consuming tissue. This demand is met by the mitochondria-rich acid producing parietal cells, which secrete the gastric acid and initiate the process of digestion. To prevent self-destruction, the columnar epithelium makes gastric mucosal barriers that resist the highly acidic and proteolytic gastric juice[26]. It is believed that mitochondrial dysfunctions impact gastric mucosal integrity, and thus measuring cellular mitochondrial O_2 consumption in gastric biopsies is justified.

Oxidative stress is induced in the stomach as a result of gastric insults, including chronic infections. GSH is a major detoxifying thiol which protects against oxidative stress. In indomethacin-treated rats, cellular GSH and mitochondrial enzymes are reduced. Esomeprazole, a proton pump inhibitor, was able to reserve GSH levels and mitochondrial enzyme activities[27]. Due to its γ-glutamyl transpeptidase, *H. pylori* can also reduce gastric epithelial GSH, exposing the bacterium, as well as the gastric epithelium, to oxidative stress[28].

ATP is produced in the mitochondria *via* oxidative phosphorylation by the proton-motive force that is used by ATP synthase to catalyze ADP phosphorylation[29]. The mitochondria are also the target of self-generated reactive oxygen species. Premalignant atrophic gastritis and gastric carcinoma are both associated with decreased respiratory capacity and mitochondrial complex I defici ency[22,30]. Therefore, investigating metabolic biomarkers in the gastric mucosa is much needed and future studies should determine whether they can be used to explore the mechanisms of diseases involving the gastric mucosa.

COMMENTS

Background

Since proliferation of gastric stem/progenitor cells and alteration of cellular dynamics are important events in carcinogenesis, the measurement of cellular bioenergetics of gastric mucosal biopsies would be an emerging need.

Research frontiers

Cellular bioenergetics has been used as a biomarker for some diseases. Whether it can be useful as a diagnostic tool for some gastric diseases is not known yet. In this study, the authors have demonstrated that various cellular bioenergetic and dynamic parameters could be measured and found useful for small gastric mucosal biopsies.

Innovations and breakthroughs

Recent reports have highlighted the importance of cellular dynamics and bioenergetics as diagnostic tools for some gastrointestinal and metabolic diseases. In this study, the authors report that cellular bioenergetics and other biochemical parameters could be useful tools for investigating stomach diseases.

Applications

By demonstrating the possible use of small mucosal biopsies for bioenergetic measurements, this study may represent a future strategy for the investigation and diagnosis of patients with upper gastrointestinal problems.

Terminology

Following microscopic examination, gastric mucosal biopsies were categorized as superficial (mild) or sever according to the Sydney classification criteria.

Peer review

This manuscript "Profiling cellular bioenergetics, glutathione levels, and caspase activities in stomach biopsies of patients with upper gastrointestinal symptoms" is very interesting study.

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ORIGINAL ARTICLE

Prospective Study

Response-guided treatment of cirrhotic chronic hepatitis B patients: Multicenter prospective study

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Abstract

AIM: To observe the effect of response-guided add-on therapy with adefovir (ADV) and lamivudine (LAM) in cirrhotic hepatitis B (CHB) patients.

METHODS: A total of 100 patients with CHB and cirrhosis were divided into three arms according to hepatitis B virus (HBV) DNA level after 24 wk LAM monotherapy: Arm A (complete response, HBV DNA $\leqslant$ 60 IU/mL, n = 49), Arm B (partial response, HBV DNA: 60-2000 IU/mL, n = 31) and Arm C (inadequate response, HBV DNA > 2000 IU/mL, n = 20). ADV was added to LAM at week 48 in Arms A and B, but at week 24 in Arm C. Virological response, YMDD mutations, biochemical response, and liver function were evaluated.

RESULTS: Comparison of the three arms demonstrated that early complete virologic response at week 24

was associated with maintained viral suppression (undetectable rate of HBV DNA at week 144 was 95.96%, 66.67% and 35.29%, respectively, P = 0.000) and reduced YMDD mutations (mutation rate at week 144 was 0%, 3.23% and 15%, respectively, P = 0.015) after 144 wk treatment. For patients who failed to achieve complete virological response at week 24, switching to combination therapy further decreased HBV DNA level by 1 $\log_{10}$ IU/mL. All three arms obtained biochemical benefits including decline of alanine aminotransferase and elevation of albumin. In patients who developed HBV DNA breakthrough for YMDD mutations, ADV add-on therapy did not induce further multiple drug resistance to LAM or ADV.

CONCLUSION: Optimized response-guided add-on therapy of ADV and LAM maintains long-term suppression of HBV DNA and improves liver function in CHB patients with compensated liver cirrhosis.

Key words: Hepatitis B; Cirrhosis; Adefovir dipivoxil; Lamivudine; Response-guided therapy

Core tip: We conducted this prospective cohort study to explore an optimized strategy of adding adefovir (ADV) and lamivudine (LAM) at different time points according to the early virological response, and to observe its association with long-term treatment outcomes in cirrhotic hepatitis B (CHB) patients with compensated cirrhosis. We found that optimized response-guided add-on therapy of ADV and LAM maintains long-term suppression of hepatitis B virus DNA and improves liver function in CHB patients with compensated liver cirrhosis.

Gu EL, Yu YQ, Wang JL, Ji YY, Ma XY, Xie Q, Pan HY, Wu SM, Li J, Chen CW, Xu XW, Wang YE, Yao GB, Wang H, Zhang WH. Response-guided treatment of cirrhotic chronic hepatitis B patients: Multicenter prospective study. *World J Gastroenterol* 2015; 21(2): 653-660 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/653.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.653

INTRODUCTION

Hepatitis B virus (HBV) remains a major public health problem, with 350-400 million people infected chronically worldwide[1]. In China, most HBV infection occurs perinatally or in early childhood, usually with a long period of immune tolerance before immune clearance[2]. Patients with cirrhotic hepatitis B are at an increased risk of major complications as disease duration extends, including hepatic cirrhosis, liver failure, and hepatocellular carcinoma (HCC). The REVEAL study in Taiwan demonstrated that an elevated level of serum HBV DNA was associated with disease progression in patients with CHB, and antiviral therapy prevented progression of liver disease[3,4]. Currently, there are several choices of nucleos(t)ide analogs (NAs) in antiviral treatment of CHB, including lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine and tenofovir disoproxil fumarate[5]. LAM was the first NA introduced into clinical use and is still a common choice for CHB patients in China as well as the Asia-Pacific region. However, administration of LAM is limited by its high rate of drug resistance, with a 3-year resistance rate of approximately 50%[6,7]. For patients with CHB and cirrhosis, the emergence of drug resistance and resumption of viral replication might lead to hepatic flares, exacerbation of liver function, or even death in some liver failure cases. Add-on ADV can effectively suppress viral replication and reduce the risk of drug resistance to LAM[8,9].

The primary aim of antiviral therapy is to suppress HBV replication and prevent disease progression. Many studies have shown that the undetectable rate of HBV DNA at week 24 was associated with reduced drug-resistance-associated mutations[10], but its predictive value for the treatment outcomes after long-term add-on ADV-LAM combination therapy has not been clarified, especially for the subgroup of CHB patients with liver cirrhosis. We conducted this prospective cohort study to explore an optimized strategy of adding ADV to LAM at different time points according to the early virological response, and to observe its association with long-term treatment outcomes in CHB patients with compensated cirrhosis.

MATERIALS AND METHODS

Study design

This prospective, multicenter cohort study was conducted at eight medical centers in China. Hepatitis B e antigen (HBeAg) positive or negative CHB patients with compensated cirrhosis were enrolled from June 2007 to Febrary 2009. All patients were given LAM 100 mg/d (Heptodin; GlaxoSmithKline China Investment Co. Ltd., Beijng, China). Patients were assigned into three arms according to serum HBV DNA levels at week 24, with Arm A $\leqslant$ 60 IU/mL (complete virological response), Arm B 60-2000 IU/mL (partial virological response), and Arm C > 2000 IU/mL (inadequate virological response). Patients in Arm C were treated with ADV 10 mg/d (Hepsera; GlaxoSmithKline China Investment Co. Ltd.) in addition to on-going LAM at week 24, while patients in Arms A and B continued LAM monotherapy until week 48, and at the end of 48 wk LAM monotherapy, ADV was added in these two arms. All the patients were monitored until week 144 (Figure 1). The study was approved by the Ethics Review Committee of Jing'an Central Hospital (Certification No. Ethic-07-05) and all the patients gave their written informed consent before enrolment in the study. The procedures were in

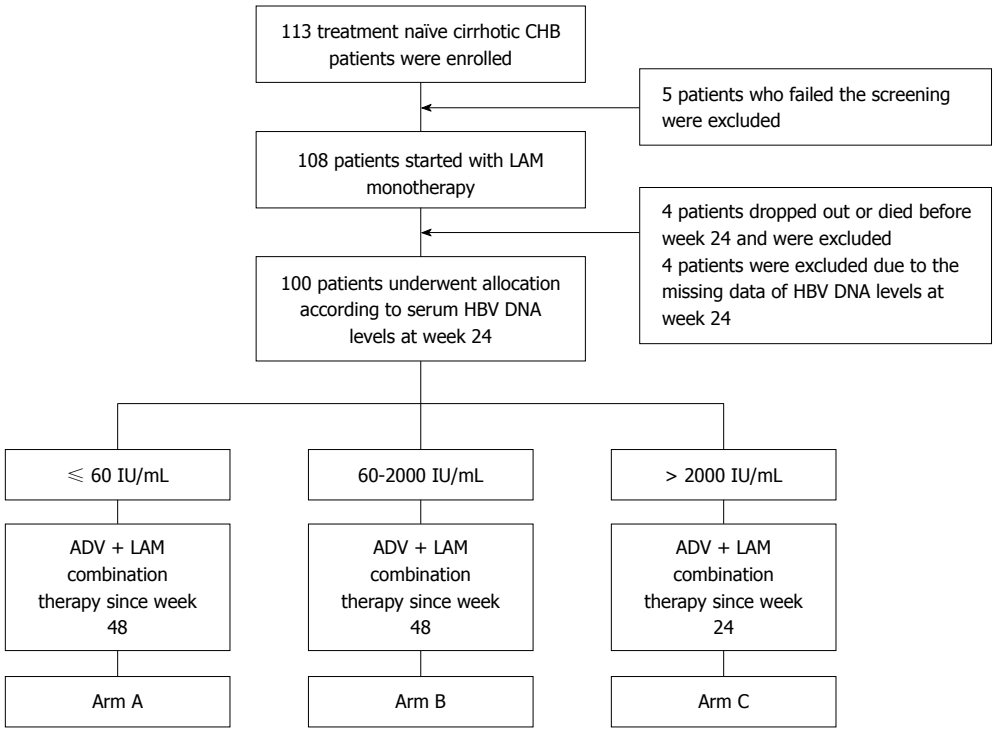

Figure 1 Study design. ADV: Adefovir dipivoxil; HBV: Hepatitis B virus; LAM: Lamivudine; CHB: Chronic hepatitis B.

accordance with the Helsinki Declaration of 1975.

Patients

Patients eligible for the study were diagnosed with compensated liver cirrhosis by clinical evidence, which was defined as platelet count < 100000/L with ultrasonographical findings suggestive of cirrhosis, including a blunted, nodular liver edge accompanied by splenomegaly (> 12 cm), or by liver biopsy showing an Ishak fibrosis score > 4[11]. Patients included in this study were Child-Pugh class A. The inclusion criteria included hepatitis B surface antigen (HBsAg) positivity for at least 6 mo; baseline HBV DNA levels > 2000 IU/mL; compensated liver cirrhosis (indicated by routine laboratory tests together with ultrasound or computed tomography results) with Child-Pugh class A; absence of co-infection with hepatitis C virus, hepatitis D virus or HIV; no previous NA treatment. Major exclusion criteria included evidence of HCC; alanine aminotransferase (ALT) > 10 times upper limit of normal; decompensated liver cirrhosis; comorbidity with other liver diseases, severe physical or mental disorders; or pregnancy.

Labortary assessment

Serum HBV DNA levels (Cobas Taqman; Roche Diagnostics Shanghai Co. Ltd., China) with the lower limit of detection (LLOD) of 12 IU/mL, liver functions [including platelet count (PLT), prothrombin time (PT), albumin, total bilirubin, ALT, aspartate aminotransferase, alkaline phosphatase and γ-glutamyl transpeptidase], and HBV serological markers (HBsAg, anti-HBs, HBeAg, anti-HBe; Abbott Architect System; Abbott China Co. Ltd., Shanghai, China) testing as well as ultrasound examination were performed every 12 wk within the first 48 wk and every 24 wk thereafter.

Definitions

Complete virological response or undetectable HBV DNA was defined as serum HBV DNA levels no more than 60 IU/mL. Virological breakthrough was defined as any increase in serum HBV DNA by > 1 $\log_{10}$ IU/mL from nadir, or redetection of serum HBV DNA at levels 10 times the LLOD after having an undetectable result[12].

Statistical analysis

Statistical analyses were performed by Stata version 11. Continuous variables were expressed as median (25-75 percentile). Categorical variables were summarized as counts and percentages. Continuous variables were compared using two-tailed Student's *t* test, analysis of variance, Mann-Whitney test, or Kruskal-Wallis test, depending on their distribution, while categorical variables were compared by χ^2 test or Fisher's exact test. Serum HBV DNA level was expressed as $\log_{10}$ IU/mL, and we regarded an HBV DNA level as 10 IU/mL when it was below LLOD for the sake of description and statistical analysis.

RESULTS

Study population

A cohort of 113 CHB patients with compensated cirrhosis were enrolled at baseline, and 100 underwent allocation according to serum HBV DNA levels at week

Table 1 Demographic data and laboratory results of the three arms at baseline

	Arm A (n = 49)	Arm B (n = 31)	Arm C (n = 20)	Statistics	P value
Median age (yr)	45 (38-52)	40 (36-50)	43.5 (40.5-55)	F = 0.8400	0.4345
Male:Female	41:8	25:6	15:5	χ^2 = 0.6979	0.7050
HBeAg-positive, n (%)	13 (26.53)	21 (67.74)[a]	14 (70)[a]	χ^2 = 17.7676	0.0000
Median PLT ($\times 10^9$/L)	109 (73-150)	109 (89-144)	98.25 (76.5-122)	χ^2 = 2.4880	0.2882
Median PT (s)	13.40 (12.6-14.3)	12.75 (12-13.7)[c]	14.30 (13-15.5)	χ^2 = 10.1270	0.0063
Median albumin (g/L)	44 (39-46.8)	43 (40-47.6)	43.5 (40.9-47.3)	F = 0.0400	0.9600
Median TB (μmol/L)	17.35 (14.5-23.35)	15.8 (13.1-20.1)	18.35 (15.9-23.6)	χ^2 = 2.1780	0.3366
Median ALT (U/L)	55 (43.2-87.5)	51 (34.5-82)	64 (48.5-91.6)	F = 0.400	0.6730
Median AST (U/L)	55 (41.05-68.5)	49 (30-57)	54.15 (45.5-66.5)	F = 1.5100	0.2251
Median HBV DNA (log10 IU/mL)	5.86 (5.16, 6.58)[c]	6.06 (5.77, 6.39)[c]	6.7 (6.29, 7.17)	F = 5.0300	0.0084

[a]P <0.05 *vs* Arm A; [c]P <0.05 *vs* Arm C. HBeAg: Hepatitis B e antigen; PLT: Platelet counts; PT: Prothrombin time; TB: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

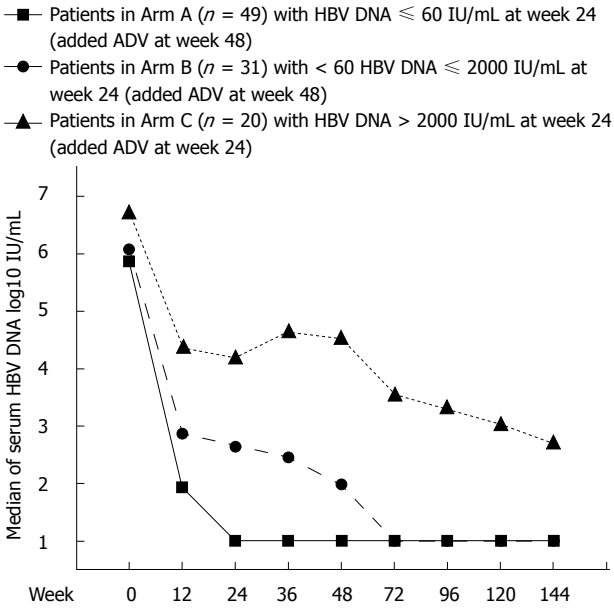

Figure 2 Pattern of hepatitis B virus DNA decline in three arms during 144 wk nucleos(t)ide analogue treatment. ADV: Adefovir dipivoxil; HBV: Hepatitis B virus.

24 (Figure 1). Age, sex ratio, and liver function results of the three arms were comparable at baseline. The proportion of HBeAg-positive CHB in Arms B and C was significantly higher than that in Arm A. Baseline HBV DNA level in Arm C was significantly higher than that in Arms A and B. The demographic characteristics of the three arms, as well as their laboratory results at baseline, are demonstrated in Table 1.

Virological response

Baseline HBV DNA levels of Arms A and B were comparable, while those in of Arm C were significantly higher at baseline as well as each time point during treatment (P < 0.05). Viral load of Arms A and B both decreased sharply after initiation of LAM monotherapy. For Arm A, serum HBV DNA levels progressively decreased until median HBV DNA level was below LLOD at week 24, and the suppression of viral replication was maintained thereafter. However, in Arm B, further reduction of HBV DNA was minimally observed after week 24 (week 24 *vs* week 48, P = 0.2059) with LAM monotherapy. After adding ADV to LAM at week 48, a reduction of approximately 1 $\log_{10}$ IU/mL of serum HBV DNA levels was resumed (week 48 *vs* week 72, P = 0.0001), and median HBV DNA level below LLOD was achieved at week 72. Serum HBV DNA level in Arm A was significantly lower than that in Arm B at each time point from weeks 12 to 144 (P < 0.05 at each time point). In Arm C, serum HBV DNA levels decreased with slow and fluctuating kinetics. After ADV was added to LAM at week 24, further reduction of approximately 1 $\log_{10}$ IU/mL serum HBV DNA levels was observed, but the median HBV DNA level of Arm C did not reach LLOD throughout treatment. Serum HBV DNA levels at each time point are depicted in Figure 2.

Undetectable HBV DNA and YMDD mutation rates at week 144

The undetectable rate of HBV DNA (≤ 60 IU/mL) at week 144 was 95.56%, 66.67% and 35.29% for Arm A, B and C, respectively (P = 0.000), as shown in Figure 3. At each time point during treatment, the differences in undetectable HBV DNA rates among the three arms were statistically significant (Figure 3). The YMDD mutation rate at week 144 was 0%, 3.23% and 15% for Arm A, B and C, respectively (P = 0.015), which is shown in Figure 4. For patients with virological breakthrough due to YMDD mutations, ADV add-on therapy did not further induce multiple drug resistance to both LAM and ADV. Early complete virological response at week 24 seemed to be associated with maintained viral suppression and reduced YMDD mutations at week 144. None of the patients who achieved complete virological response at week 24 (Arm A) developed YMDD mutation at week 144. For patients who failed to achieve complete virological response at week 24, undetectable rate of HBV DNA was increased after switching to LAM and ADV combination therapy, but still far from

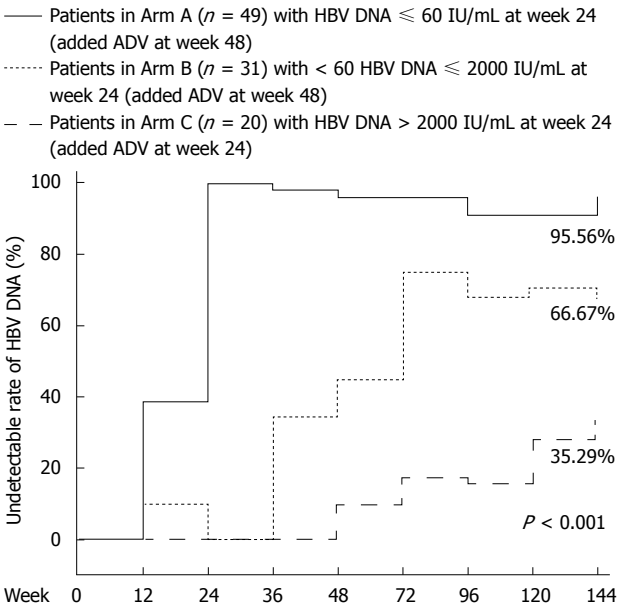

Figure 3 Undetectable rates of serum hepatitis B virus DNA at different time points in three arms. ADV: Adefovir dipivoxil; HBV: Hepatitis B virus.

satisfactory when compared with Arm A.

HBeAg loss and HBeAg seroconversion

The number of patients with HBeAg-positive CHB at baseline was 13, 21 and 14 for Arm A, B and C, respectively. At week 144, HBeAg loss rate was 53.85% (7/13), 47.62% (10/21) and 42.86 (6/14) ($P = 0.993$), and HBeAg seroconversion rate was 23.08% (3/13), 47.62% (10/21) and 21.43% (3/14) ($P = 0.245$) in the three arms, respectively (Figure 4).

Biochemical response and changes in liver function

Biochemical response and improvement of liver function were achieved after NA treatment, and no biochemical breakthrough was observed during 144-wk follow-up. There was no significant difference in PLT and PT at week 48 compared to baseline in each arm (Figure 5A and B). Serum ALT levels decreased significantly at week 48, which reduced from 55 (43.2-87.5) U/L to 31.75 (24.5-43.1) U/L ($P = 0.0000$) and from 51 (34.5-82) U/L to 35.8 (26-50) U/L ($P = 0.0092$) in Arm A and B, respectively. For Arm C, reduction of serum ALT levels occurred after LAM and ADV combination therapy [week 0 *vs* 48: 64 (48.5-91.6) U/L *vs* 49.3 (29.5-82) U/L, $P = 0.2471$; week 0 *vs* 96: 64 (48.5-91.6) U/L *vs* 39.55 (26.9-52) U/L, $P = 0.0130$] (Figure 5D). For the parameters of liver function, the increase in serum levels of albumin was the most remarkable. At week 48, serum levels of albumin rose from 44 (39-46.75) g/L to 47 (43.9-48.65) g/L in Arm A ($P = 0.0006$), 43 (40-47.6) g/L to 47 (43.4-49) g/L in Arm B ($P = 0.0029$), and 43.5 (40.9-47.3) g/L to 46 (45.2-48.25) g/L in Arm C ($P = 0.0045$) (Figure 5C). Biochemical results of the three arms were comparable at each time point.

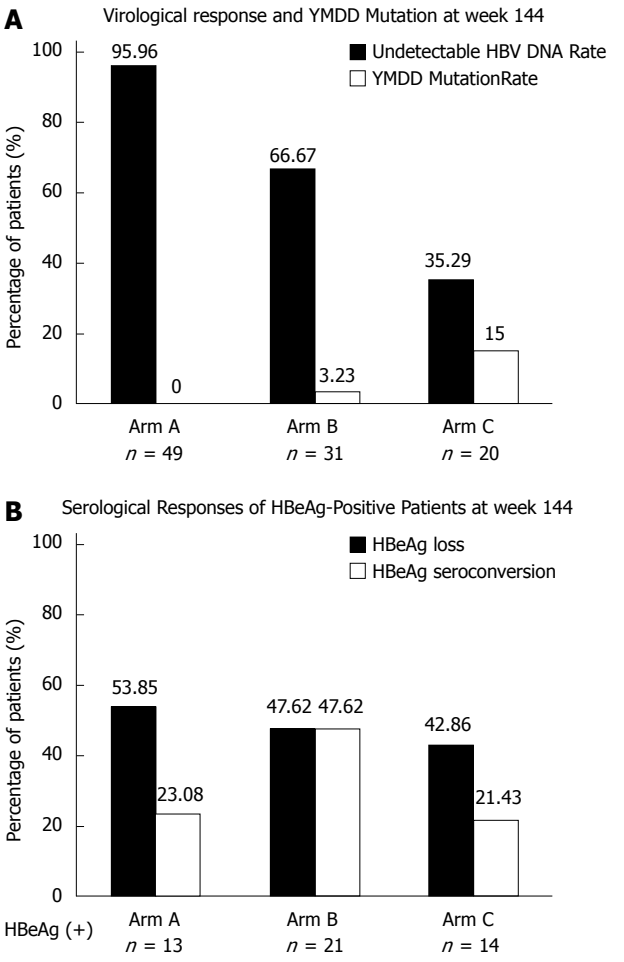

Figure 4 Undetectable rates of serum hepatitis B virus DNA levels, YMDD mutation rates, hepatitis B e antigen loss and hepatitis B e antigen seroconversion rates at week 144 in three arms. A: Virological response and YMDD Mutation at week 144; B: Serological responses of hepatitis B e antigen (HBeAg)-positive patients at week 144.

DISCUSSION

Several clinical studies have suggested that ADV add-on therapy is associated with a higher rate of virological response and reduced antiviral resistance, compared with sequential montherapy with LAM and ADV[8,9,13,14] in patients with CHB. According to the roadmap concept proposed by Keeffe *et al*[15], assessment of virological response at week 24, which is predictive of long-term treatment outcomes, is of significant importance for further treatment decisions. However, no prospective study has been reported to evaluate the response-guided strategy of ADV add-on therapy to LAM-based treatment in CHB patients with compensated cirrhosis. We conducted this multicenter, prospective cohort study to explore the optimal strategy of ADV add-on LAM combination therapy in the specified subgroup of CHB patients with cirrhosis, using the roadmap concept.

All the patients started with LAM monotherapy and had ADV added at different times, according to the virological response at week 24. The total treatment

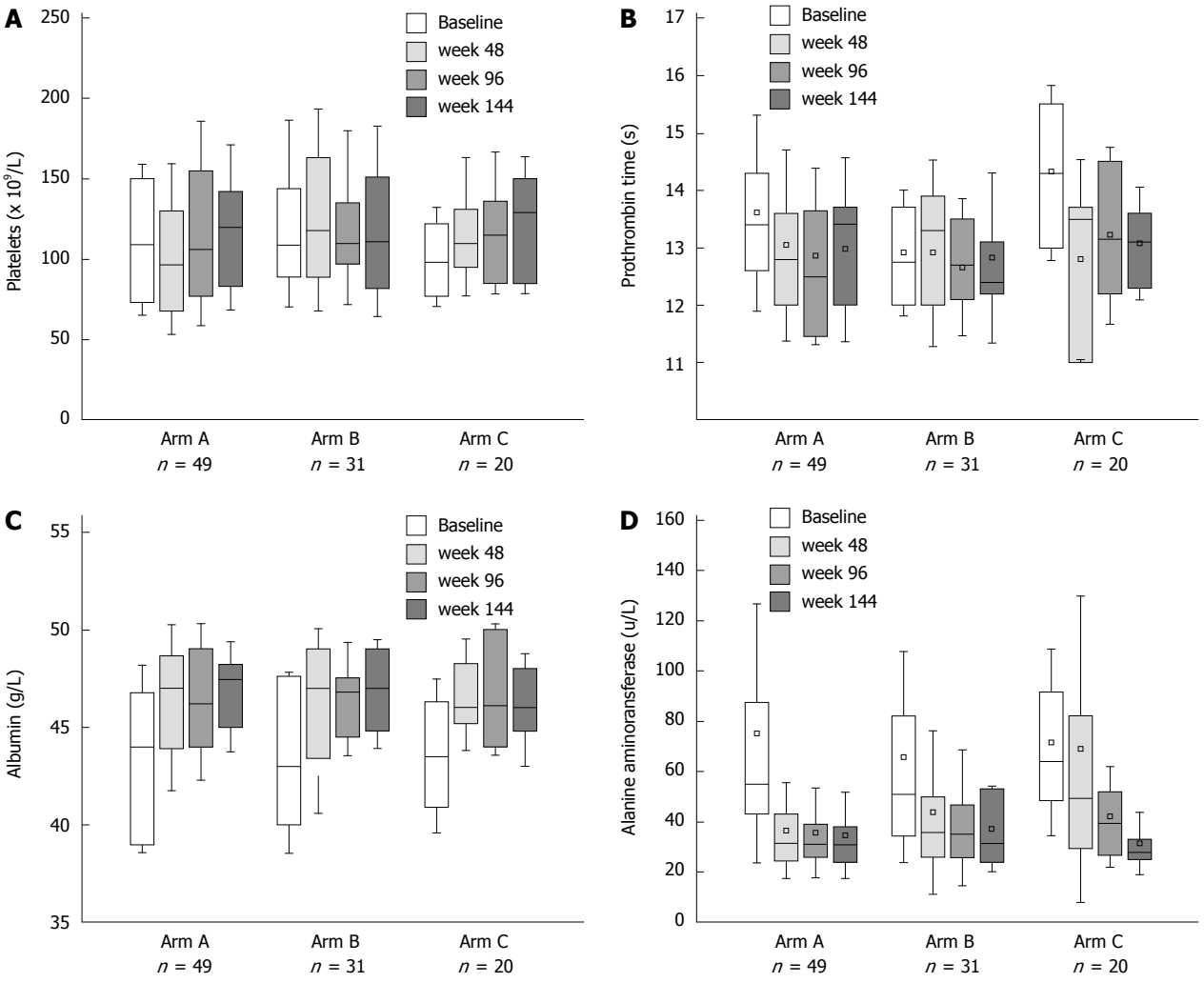

Figure 5 Changes after nucleos(t)ide analogue treatment in three arms. A: Platelet counts; B: Prothrombin time; C: Albumin; D: Alanine aminotransferase.

duration of the study was 144 wk. Our findings indicated that long-term antiviral therapy was effective in suppressing HBV replication, achieving serological and biochemical responses, and improving liver functions in CHB patients with compensated cirrhosis. Early virological response was associated with sustained viral suppression and a lower rate of drug resistance during long-term treatment[16,17].

Early ADV add-on can prevent the emergence of resistance to LAM[18]. In this study, add-on therapy of ADV to LAM was conducted even in patients with complete response after week 48. In the Asia-Pacific region, especially in economically undeveloped areas, LAM is still the first option for CHB and HBV-related cirrhosis. The resistance rate was 24% after 1 year LAM treatment and rose to 70% after 5 years treatment[6,19]. Emergence of resistance may exacerbate disease, reduce the benefit of antiviral therapy, and even threaten the life of patients with liver cirrhosis. Therefore, add-on therapy of ADV to LAM should be conducted even in patients with a complete response. For patients with compensated cirrhosis who achieved a complete virological response at week 24, the risk of drug resistance was reduced significantly by prolonged treatment. ADV add-on LAM combination therapy could maintain virological response and prevent the emergence of drug resistance, with an undetectable rate of HBV DNA of 95.96% and none of the patients developed YMDD mutations at week 144.

The ADV add-on strategy was also considered effective for patients with partial virological response at week 24. Further reduction of HBV DNA levels was observed and most of the patients with compensated cirrhosis achieved sustained undetectable HBV DNA after week 72. In addition, the YMDD mutation rate at week 144 was only 3.23%. However, serum HBV DNA levels did not further decrease with the continuation of LAM monotherapy from weeks 24 to 48, which suggests that immediate add-on of ADV at week 24 might be beneficial for patients with partial response.

After long-term therapy, patients in Arm C with inadequate virological response at week 24 achieved significantly lower rates of undetectable HBV DNA at week 144 than did patients in Arms A and B, even though combination therapy was initiated at an earlier stage. Despite a reduction of approximately 1 $\log_{10}$ IU/mL of serum HBV DNA after combination therapy, the median

HBV DNA level did not reach LLOD throughout treatment. Moreover, the YMDD mutation rate at week 144 was 15%, which was significantly higher than that of Arms A and B. Thus, for patients with inadequate virological response at week 24, switching to more potent antiviral agents with a high genetic barrier and without cross-resistance to LAM would be a better choice[20].

In conclusion, most CHB patients with compensated liver cirrhosis benefited from long-term antiviral therapy. Significant improvement of liver function was observed in all patients, regardless of the degree of HBV DNA reduction. Early virological response at week 24 was associated with satisfactory long-term treatment outcomes and was valuable for individualized treatment decisions. To avoid HBV DNA breakthrough, add-on therapy of ADV to LAM was effective in maintaining viral suppression and reducing YMDD mutations in patients with complete or partial virological response. Meanwhile, switching to more potent antiviral monotherapy or combination therapy was suggested for patients with inadequate response at week 24.

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COMMENTS

Background

Many studies have shown that the undetectable rate of hepatitis B virus (HBV) DNA at week 24 was associated with reduced drug-resistance-associated mutations. According to the roadmap concept, assessment of virological response at week 24, which was predictive of long-term treatment outcomes, was of significant importance for further treatment decisions. However, no prospective study has been reported to evaluate the response-guided strategy of adefovir (ADV) add-on therapy to lamivudine (LAM)-based treatment in chronic hepatitis B (CHB) patients with compensated cirrhosis.

Research frontiers

LAM can achieve sustained viral suppression, slow down progression of liver disease, and prevent development of major complications or hepatocellular carcinoma. However, long-term LAM may induce selective drug-resistant mutations, reduce the clinical benefit, and lead to exacerbation of liver functions, or even death. The incidence of viral breakthrough was 5.1%-36.0% in LAM-resistant CHB patients treated with entecavir (ETV), compared to 0%-3% in those receiving LAM plus ADV combination therapy. Therefore, ADV add-on therapy seemed to achieve more sustained suppression of viral replication than sequential ETV monotherapy in LAM-resistant patients. Considering the fact that tenofovir disoproxil fumarate has not come to market and the reality of the economic situation in China, ADV add-on therapy is still likely to be the optimal choice for LAM-resistant patients for the foreseeable future.

Innovations and breakthroughs

Several clinical studies have suggested that ADV add-on strategy is associated with a higher rate of virological response and reduced antiviral resistance, compared with sequential monotherapy of LAM and ADV in patients with CHB. However, few prospective studies have been reported to evaluate the response-guided strategy of ADV add-on therapy to LAM-based treatment in CHB patients with compensated cirrhosis. This was a multicenter, prospective cohort study to explore the optimal strategy of ADV add-on LAM combination therapy in the specified subgroup of cirrhotic CHB patients, using the roadmap concept.

Applications

In the Asia-Pacific region, especially in the economically undeveloped areas, LAM is still the first option for CHB and HBV-related cirrhosis. Nucleot(s)ide analogs with a low-resistance barrier represent the first-line therapy for 65% of patients in China. The study was of importance for choosing the optimal strategy for response-guided treatment to avoid drug resistance in CHB patients with compensated cirrhosis.

Terminology

Complete virological response or undetectable HBV DNA was defined as serum HBV DNA levels no more than 60 IU/mL. Virological breakthrough was defined as any increase in serum HBV DNA by > 1 $\log_{10}$ IU/mL from nadir or redetection of serum HBV DNA at levels 10 times the lower limit of detection after having an undetectable result.

Peer review

This type of study may provide important information or guidelines to treat CHB patients with compensated liver cirrhosis.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Seven-day quintuple regimen as a rescue therapy for *Helicobacter pylori* eradication

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Author contributions: Mansour-Ghanaei F, Naghipour MR and Joukar F designed the study; Joukar F supervised the procedure and analyzed the data; Forouhari A collected data; Mansour-Ghanaei F, Joukar F and Seyed Saadat SM wrote the manuscript; all authors approved the final manuscript.
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Abstract

AIM: To determine the efficacy of two quintuple regimens for eradication of *Helicobacter pylori* (*H. pylori*) in patients who failed previous therapies.

METHODS: This prospective, open-label, randomized controlled trial was a phase Ⅱ study conducted from April 2011 to March 2012 at the Gastrointestinal and Liver Diseases Research Center in Rasht, Iran. A total of 208 patients with dyspepsia who failed previous *H. pylori* eradication with a ten-day quadruple therapy were enrolled. A random block method was used to assign patients to one of two treatment groups. Patients in the first group were treated with 240 mg bismuth subcitrate, 20 mg omeprazole, 1000 mg amoxicillin, 500 mg clarithromycin and 500 mg tinidazole (BOACT group). Patients in the second group received a regimen containing 240 mg bismuth subcitrate, 20 mg omeprazole, 500 mg tetracycline, 500 mg metronidazole and 200 mg ofloxacin (BOTMO group). Both regimens were given twice daily for a duration of seven days. The eradication was confirmed by a ^{14}C urea breath test 12 wk after completion of therapy. Patient compliance and drug side effects were evaluated at the end of the treatment period. The success rates were calculated by intention-to-treat and per-protocol analyses.

RESULTS: A total of 205 patients completed the course of treatment, with three patients excluded due to drug intolerance. The mean age of patients did not differ between the BOACT and BOTMO groups (41.6 ± 12.2 years *vs* 39.6 ± 11.8 years), and no significant differences were found between the two groups in terms of age, sex, smoking habits or the initial eradication regimen. The intention-to-treat and per-protocol eradication rates were significantly higher in the BOTMO group (86.5%, 95%CI: 0.85-0.87 and 86.7%, 95%CI: 0.80-0.89, respectively) compared with the BOACT group (75.5%, 95%CI: 0.73-0.76 and 76%, 95%CI: 0.69-0.80, respectively) ($P < 0.05$). Univariate analyses for both groups did not show any association of sex, smoking and initial therapeutic regimen with

eradiation rate (P > 0.05 for all). Significantly more patients experienced side effects in the BOACT group compared to the BOTMO group (77.4% *vs* 36.6%, P < 0.01). This difference was exemplified by increases in headache and taste disturbance (P < 0.05).

CONCLUSION: Quintuple therapy with a BOTMO regimen is an alternative second-line rescue therapy for Iranian patients with failed first-line eradication treatment of *H. pylori*.

Key words: Antibiotic resistance; Rescue therapy; Eradication; *Helicobacter pylori*; Quintuple therapy

Core tip: Due to increasing antibiotic resistance, eradication of *Helicobacter pylori* has become more challenging. Antibiotic resistance exhibits a regional pattern and treatments typically involve 14-d medication periods, which are not always effective. This study compared two 7-d quintuple regimens and identified a regimen of bismuth subcitrate, omeprazole, tetracycline, metronidazole, and ofloxacin as an effective alternative second-line rescue therapy with minimal side effects for Iranian patients who failed a course of first-line treatment.

Mansour-Ghanaei F, Joukar F, Naghipour MR, Forouhari A, Seyed Saadat SM. Seven-day quintuple regimen as a rescue therapy for *Helicobacter pylori* eradication. *World J Gastroenterol* 2015; 21(2): 661-666 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/661.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.661

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is a global health problem associated with chronic gastritis, peptic ulcer disease and gastric cancer, which affects 20%-50% of people in Western nations and up to 80% of the population in developing countries[1,2]. Therefore, the eradication of the pathogen is of great importance to reduce *H. pylori*-related complications[3,4]. However, treatment failures resulting from antimicrobial resistance and poor compliance have become an increasing concern. This is especially important in regions with a high prevalence of *H. pylori* infection, such as Iran, where the prevalence, re-infection rate and resistance to standard therapeutic regimens are much higher than in Western countries[4-7]. Treatment with triple therapy, which is the most frequently recommended, fails to eradicate *H. pylori* in approximately 20% of cases[8]. Treatment with quadruple rescue therapy is still insufficient to reduce the failure rate below 20%[9,10]. Bacterial culture and microbial susceptibility tests are recommended by European guidelines for selection of third-line treatment regimens, but these methods are hindered by low sensitivity, high cost, unavailability and their invasive nature[11,12]. Therefore, designing a novel rescue regimen that achieves greater than 80% eradication rate is a target of current research[11,13].

Recently, several multidrug rescue regimens against refractory *H. pylori* infection have been studied, though an ideal therapeutic regimen has not yet been identified[12,14-18]. In Iran, the most common regimen for the first-line treatment is a 14-d quadruple therapy containing bismuth subcitrate, omeprazole, metronidazole and either tetracycline or amoxicillin[19]. Mousavi *et al*[20] showed that a 14-d quadruple therapy (including amoxicillin) resulted in an eradication rate of 70.4% based on an intention-to-treat (ITT) analysis, and 75.7% based on a per-protocol (PP) analysis. Similarly, Agah *et al*[5] reported a 68% eradication rate using the same regimen. A higher eradication rate of 84% by ITT analysis was reported by Fakheri *et al*[21] with quadruple therapy including *bismuth subcitrate, omeprazole, amoxicillin* and *clarithromycin*. Despite the benefit, clarithromycin exhibits resistance that varies over time and based on the geographic region. In Iran, there is a high prevalence of clarithromycin and metronidazole resistance, indicating that Western eradication regimens are not ideal in this region[22]. Our previous study in an antibiotic-sensitive area of Iran using 7- and 14-d furazolidone-based quadruple regimens failed to show acceptable eradication rates by ITT analysis (71% and 65%, respectively)[23]. Therefore, rescue regimens should be chosen based on the regional pattern of antibiotic resistance, taking into account patient compliance, drug efficacy and safety[5,22]. The aim of this study was to compare two quintuple rescue therapy regimens with regard to compliance, safety and efficacy in patients who had failed an initial quadruple course of therapy.

MATERIALS AND METHODS

Setting

This phase Ⅱ study was a prospective, open-label, randomized controlled trial conducted from April 2011 to March 2012 at the Gastrointestinal and Liver Diseases Research Center of Guilan University of Medical Sciences, in Rasht, Iran. The study was approved by the ethics committee of the research center, and was in accordance with the Helsinki declaration for use of human subjects. This study is registered in the Iranian Registry of Clinical Trials (identification number: IRCT201103011155N11, Available from: URL: http://www.irct.ir).

Participants

Patients with *H. pylori* infection who failed previous eradication with a ten-day quadruple therapy comprised of bismuth subcitrate, omeprazole, amoxicillin and clarithromycin or bismuth subcitrate, omeprazole, amoxicillin and metronidazole were consecutively recruited for this study (n = 208). The patients were referred from the outpatient gastroenterology clinics

and private offices to our referral University center. Twelve weeks after completion of therapy, the diagnosis of *H. pylori* infection was made using a Heliprobe ^{14}C urea breath test (Kibion AB, Uppsala, Sweden), which shows 94% sensitivity and 100% specificity[24]. Patients under 15 or over 65 years of age, and those with co-existing serious illnesses such as liver cirrhosis, uremia and gastrointestinal malignancies were excluded from the study. Other exclusion criteria were pregnancy/lactation and having contraindication or allergy to any of the study drugs. The objectives of the study and potential side effects of drugs were explained to each patient, and informed written consent was obtained.

Randomization

Patients were randomized according to classification guidelines of the Federal Drug Administration/World Health Organization for individually randomized trials for the testing of drugs or devices[25]. The random block method was used to assign patients into randomly permuted treatment blocks to ensure an equal number of subjects for each treatment. The first group consisted of 104 patients who received 240 mg bismuth subcitrate, 20 mg omeprazole, 1000 mg amoxicillin, 500 mg clarithromycin and 500 mg tinidazole twice daily for seven days (BOACT group). The second group of 104 patients was treated with 240 mg bismuth subcitrate, 20 mg omeprazole, 500 mg tetracycline, 500 mg metronidazole and 200 mg ofloxacin twice daily for seven days (BOTMO group). Demographic and clinical variables, including age, sex, smoking status and type of previous treatment regimen, were recorded. Patients were instructed to take their prescribed medications at the scheduled times and advised to avoid smoking, drinking alcoholic or caffeinated beverages, eating spicy foods or taking non-steroidal anti-inflammatory drugs or medications containing a monoamine oxidase inhibitor.

Outcomes

The primary outcome measured was the *H. pylori* eradication rate as assessed by the ^{14}C urea breath test. Successful eradication of *H. pylori* was confirmed by a negative result. The secondary outcomes were the incidence of adverse effects and patient compliance. Adverse effects from the treatments were assessed using a 0-10 scale system (mild: 0-3, moderate: 4-6, severe: 7-10), and patient compliance was defined as a consumption of > 80% of the prescribed drugs.

Statistical analysis

ITT and PP analyses were performed to assess the efficacy of the treatment regimens for *H. pylori* eradication. The ITT analysis included all patients who were initially randomized into one of the treatment groups and took at least one treatment dose. The PP analysis excluded patients who refused to continue the treatment, or those with poor compliance to therapy. *H. pylori* eradication percentages, odds ratios and 95%CI were assessed for each group. Demographic variables, previous treatments, eradication rates, adverse events and patient compliance were compared between the groups using χ^2 and Student's *t* analyses. Statistical analyses were performed using SPSS, version 16.0 software (SPSS Inc., Chicago, IL, United States), and $P < 0.05$ was considered to be statistically significant.

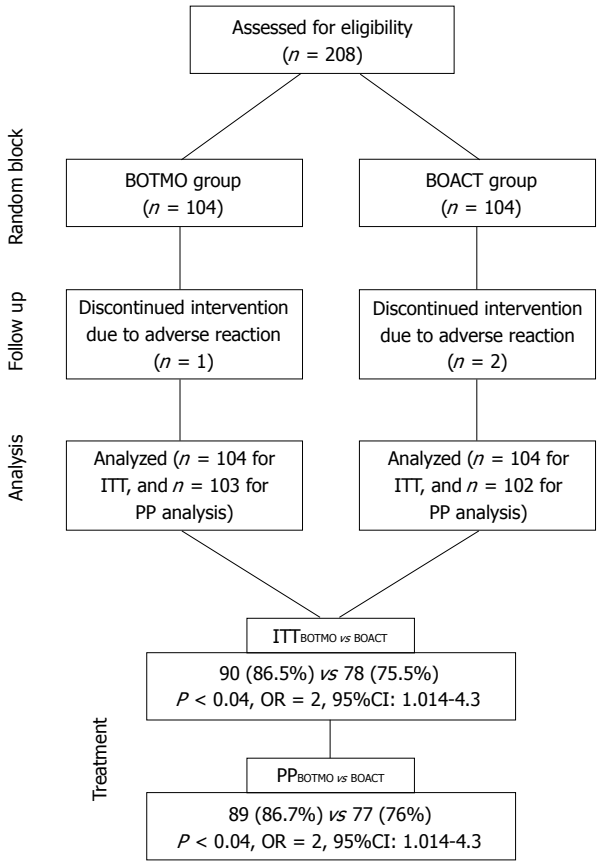

Figure 1 Flow diagram of quintuple therapy comparisons. BOACT: Bismuth subcitrate, omeprazole, amoxicillin, clarithromycin, tinidazole; BOTMO: Bismuth subcitrate, omeprazole, tetracycline, metronidazole, ofloxacin; ITT: Intention-to-treat; OR: Odds ratio; PP: Per-protocol.

RESULTS

A total of 208 patients with persistent *H. pylori* infection were enrolled in this study. Of the 104 assigned to each group, two patients (females with severe epigastric pain and headache) in the BOACT group and one patient (male with severe nausea) in the BOTMO group were excluded from the study due to drug intolerance (Figure 1).

Basic demographic and clinical characteristics of the study population and their initial eradication therapy regimen are shown in Table 1. The mean age was 41.6 ± 12.2 years for patients treated with BOACT and 39.6 ± 11.8 years for those receiving the BOTMO regimen. Most of the patients were female (BOACT: 72/104, 69.2%; BOTMO: 65/104, 62.5%). No significant differences were found between the two groups in terms of age, sex, smoking habits or initial regimen.

Table 1 Baseline demographic and clinical characteristics

Characteristic	BOACT (n = 104)	BOTMO (n = 104)
Male/Female	32/72	39/65
Age (yr)	41.6 ± 12.2	39.6 ± 11.8
Smoking, n (%)		
Yes	28 (26.9)	27 (26.0)
No	76 (73.1)	77 (74.0)
Initial eradication regimen, n (%)		
BOAC	82 (78.8)	75 (72.1)
BOAM	22 (21.2)	29 (27.9)

BOAC: Bismuth subcitrate, omeprazole, amoxicillin, clarithromycin; BOACT: Bismuth subcitrate, omeprazole, amoxicillin, clarithromycin, tinidazole; BOAM: Bismuth subcitrate, omeprazole, amoxicillin, metronidazole; BOTMO: Bismuth subcitrate, omeprazole, tetracycline, metronidazole, ofloxacin.

Table 2 Reported side effects n (%)

Side effect	Regimen	
	BOACT (n = 102[1])	BOTMO (n = 103[2])
Headache	17 (17.6)[a]	7 (7.8)
Taste disturbance	14 (15.7)[a]	6 (5.8)
Nausea	5 (4.9)	3 (2.9)
Epigastric pain	4 (3.9)	2 (1.9)
Diarrhea	4 (3.9)	2 (1.9)
Heartburn	3 (2.9)	2 (1.9)
Stool color change	3 (2.9)	2 (1.9)
Urine color change	2 (1.9)	1 (0.9)
Anorexia	3 (2.9)	1 (0.9)
Total	55 (77.4)[b]	26 (36.6)

[1]Two and [2]one of the patients from the group discontinued treatment due to severe adverse effects. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control. BOACT: Bismuth subcitrate, omeprazole, amoxicillin, clarithromycin, tinidazole; BOTMO: Bismuth subcitrate, omeprazole, tetracycline, metronidazole, ofloxacin.

Eradication of H. pylori

On ITT analysis, the eradication rate was 75.5% (95%CI: 0.73-0.76) in the BOACT group and 86.5% (95%CI: 0.85-0.87) in the BOTMO group; the difference between the two groups was statistically significant (OR = 2, 95%CI: 1.014-4.300; $P < 0.04$). In the PP analysis, *H. pylori* was eradicated in 76% of patients in the BOACT group (95%CI: 0.69-0.80) and 86.7% of patients in the BOTMO group (95%CI: 0.80-0.89); the difference between the two groups was statistically significant (OR = 2, 95%CI: 1.014-4.300; $P < 0.04$).

Univariate analyses for both groups did not show any association of sex, smoking and initial therapeutic regimen with eradiation rate ($P > 0.05$ for all).

Compliances and adverse effects

Despite the discontinuation of treatment by two patients in the BOACT group and one patient in the BOTMO group, both regimens were well-tolerated by the majority of patients. A total of 71 side effects were reported in 59 patients (28.8%), which were rated as mild (Table 2). A significantly greater proportion of patients reported adverse side effects in the BOACT group compared to the BOTMO group (77.4% *vs* 36.6%; $P < 0.01$). This corresponded with 55 side effects in 35 BOACT patients and 26 side effects in 20 BOTMO patients. Specifically, significantly more reports of headache and taste disturbance occurred in the BOACT group than in the BOTMO group ($P < 0.05$).

DISCUSSION

The results of the present study show a higher *H. pylori* eradication rate with a 7-d quintuple therapy with BOTMO compared to BOACT in patients who initially failed quadruple therapies. Although both regimens demonstrated good patient compliance, fewer side effects were reported in patients receiving BOTMO therapy. These findings are consistent with those of the only other study comparing the efficacy of a quintuple regimen, comprised of bismuth subcitrate, tetracycline, metronidazole, roxithromycin and lansoprazole, with triple and quadruple treatment regimens[26]. In that study, a significantly higher rate of *H. pylori* eradication was found with the quintuple regimen, though the length of treatment was 14 d and side effects were not evaluated.

At the present, triple therapy suggested by both Canadian and European guidelines is the most preferred first-line regimen in clinical practice[3,27]. However, the success rate of this eradication regimen is decreasing[10,17]. Even the most commonly recommended quadruple rescue therapy regimen fails to eradicate infection in more than 20% of patients[6,28]. In one study of patients with peptic ulcers who failed to respond to previous eradication regimens, an eradication rate of 69% was obtained after treatment with a 7-d course of therapy with bismuth subcitrate, a high-dose of furazolidone (200 mg, b.i.d), amoxicillin and a proton-pump inhibitor[29]. A similar eradication rate (63% by ITT analysis) was achieved in another study using a 7-d rescue quadruple regimen containing bismuth subcitrate, omeprazole, tetracycline and a high-dose of furazolidone (200 mg, b.i.d)[30].

Iranian patients show an increasing resistance to metronidazole, clarithromycin[5,22,23] and furazolidone[23]. In order to overcome the challenge of *H. pylori* eradication failure, several maiden rescue regimens have recently been proposed[14,16,18]. Furthermore, Sardarian *et al*[31] compared the efficacy of a hybrid therapy (40 mg pantoprazole and 1000 mg amoxicillin for 14 d, with 500 mg clarithromycin and 500 mg tinidazole for the last 7 d, b.i.d) with sequential therapies (40 mg pantoprazole for 10 d with 1000 mg amoxicillin for the first 5 d and 500 mg clarithromycin and 500 mg tinidazole for the last 5 d, all twice daily) for *H. pylori* eradication in 396 Iranian patients[31]. The rates of compliance were 96.7% and 98.6% for the hybrid and sequential groups, respectively. The eradication rate for the hybrid group was significantly higher than that of the sequential group by both ITT (89.5% *vs* 76.7%) and PP (92.9% *vs* 79.9%) analyses. Severe side effects were observed in 2.4% of patients in the hybrid group and 3.8% of those in the sequential group.

According to the results of our study, the quintuple BOTMO regimen was successful in eradicating *H. pylori* in 86.5% and 86.7% of patients by ITT and PP analyses, respectively. Although a cure rate of > 80% was achieved, which is acceptable by the standards of Maastricht and other guidelines for successful eradication[32], none of the regimens achieved the target threshold for an ideal eradication regimen of more than 90%. It is possible that the efficacy of the clarithromycin-based BOACT regimen used in the present study was affected by the use of clarithromycin in the failed initial eradication therapies.

In addition to being effective and compatible with regional microbial resistance patterns, a suitable anti-*H. pylori* regimen should be cost-effective, easy to administer and well-tolerated[3,18]. In the present study, approximately one-third of patients experienced adverse events, which were reported as mild to moderate. Of the total study population, only three patients discontinued treatment due to severe side effects. Generally, both treatment regimens were well-tolerated and had a good compliance (98.7% *vs* 99.04% in BOACT and BOTMO regimens, respectively).

A limitation of this study was the lack of regional estimates of eradication rates with regard to antibiotic resistance. Furthermore, the results of this study may not be applicable to patients who failed other therapies. *H. pylori* is an actively dividing spiral bacterium that assumes a coccoid morphology under stressful conditions such as antibiotic exposures[33-35], which could contribute to treatment failures and relapse of infection[35-38]. Faghri *et al*[35] suggested that a therapy must eradicate viable coccoids in addition to the spiral forms, in order to be successful.

In conclusion, quintuple rescue therapy using a BOTMO regimen provided higher eradication rates than the BOACT regimen. Furthermore, the drugs used in the BOTMO regimen induced fewer side effects and are widely available in regions of Iran where culturing of *H. pylori* is difficult. Thus, the BOTMO regimen could be an alternative second-line rescue therapy for Iranian patients who failed previous eradication treatment. However, the regional pattern of antimicrobial resistance necessitates that more studies in other populations be conducted. Moreover, treatment regimens of longer than seven days should also be evaluated.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is associated with chronic gastritis, peptic ulcer disease and gastric cancer. Therefore, the eradication of the pathogen is of great importance in order to reduce *H. pylori*-related complications. As no new drugs to treat *H. pylori* have been developed, eradication requires multiple-drug therapies. If a drug regimen fails to eradicate the bacteria, an appropriate second-line therapy should be selected.

Research frontiers

H. pylori resistance to antibiotics is the most important factor in treatment failure. This necessitates the development of new, alternative protocols for successful treatment.

Innovations and breakthroughs

This is the first study to evaluate the efficacy of two seven-day quintuple rescue regimens including bismuth subcitrate, omeprazole and either amoxicillin, clarithromycin and tinidazole, or tetracycline, metronidazole and ofloxacin as a second-line treatment for *H. pylori* following the failure of first-line regimens in Iranian patients.

Applications

This study indicates that quintuple therapy with bismuth subcitrate, omeprazole, tetracycline, metronidazole and ofloxacin for seven days is an effective alternative second-line rescue therapy for Iranian patients who failed first-line treatment of *H. pylori* infection.

Terminology

Quintuple therapy for *H. pylori* eradication involves treatment with bismuth subcitrate, three antibiotics and a proton-pump inhibitor.

Peer review

This study provides useful information and suggestions for future research evaluating treatment regimens for *H. pylori* eradication. The authors show that tetracycline-containing quintuple rescue therapy is highly effective in treating *H. pylori* eradication failures of first-line regimens in Iran.

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Biopathologic features and clinical significance of micrometatasis in the lymph node of early gastric cancer

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Abstract

AIM: To evaluate the biopathologic features and clinical significance of nodal micrometastasis (MI) in early gastric cancer (EGC).

METHODS: Among 1022 EGC patients who underwent gastrectomy with lymphadenectomy of D1 + β or more from March 2001 to December 2005 at the Korean National Cancer Center, available nodal metastasis was found in 90 pT1N1 patients. Nodal metastasis was confirmed by immunohistochemistry (IHC) with cytokeratin and patients were classified into MI and macrometastasis (MA) groups based on the main tumor burden according to the 6th International Union Against Cancer/American Joint Committee on Cancer staging system; the main tumor burden with a diameter of greater than 0.2 mm but no greater than 2 mm as MI, and greater than 2 mm as MA of the representative metastatic node. Proliferative and apoptotic activities of the primary tumor and the nodal metastasis were measured by IHC with Ki-67 and terminal deoxynucleotidyl transferase dUTP nick end labeling, respectively. Biopathologic and clinical features of the patients were analyzed and compared between MI and MA groups. Patients with recurrence were compared with those without recurrence to identify risk factors for recurrence.

RESULTS: Thirty-seven patients showed MI and the other 53 patients revealed MA in the lymph node; the incidence of patients with MI and MA was 41.1% and 58.9%. The main tumor burden was 0.9 and 4.6 mm in the representative metastatic node, respectively. Japanese N2 stations were more frequently involved in MA group (20.9%) than in MI group (10.3%) but

the difference was not statistically different (P = 0.338). Proliferative and apoptotic activities of MI were decreased than those of MA (26.7% *vs* 40.5%, P = 0.004 and 1.0% *vs* 3.0%, P < 0.001, respectively). However, nodal MI in the current study showed a relatively high proliferative activity and an equivalent apoptotic activity compared to other cancers in the previously published studies. Recurrence was observed in 6 patients during the mean follow up period of 87.6 ± 26.2 mo. The recurrence was significantly associated with the presence of MA (P = 0.041) and lymphovascular invasion of the primary tumor (P = 0.032).

CONCLUSION: Lymphadenectomy of D1 + β or more might be necessary in patients with MI in sentinel node to prevent recurrence by clearing MI involving Japanese N2 station.

Key words: Early gastric cancer; Sentinel node biopsy; Lymphadenectomy; Micrometastasis; Macrometastasis

Core tip: Nodal micrometastasis in early gastric cancer (EGC) has a relatively high proliferative and an equivalent apoptotic activities compared to other cancers. The incidence of Japanese N2 station micrometastasis involvement is about 10%. Lymphadenectomy of D1+β or more might be necessary if micrometastasis is identified during sentinel node biopsy in EGC.

Jo MJ, Park JY, Song JS, Kook MC, Ryu KW, Cho SJ, Lee JH, Nam BH, Hong EK, Choi IJ, Kim YW. Biopathologic features and clinical significance of micrometatasis in the lymph node of early gastric cancer. *World J Gastroenterol* 2015; 21(2): 667-674 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/667.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.667

INTRODUCTION

Nodal metastasis is the one of the important prognostic factors as along with the depth of invasion of the primary tumor and distant metastasis in solid cancers. Nodal metastasis is classified into isolated tumor cell (ITC), micrometastsis (MI) and macrometastasis (MA) depending on the size of metastatic deposit in the lymph node according to the 6th edition of International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) staging system[1]. This classification system was developed through histological examinations, such as immunohistochemisty (IHC), of melanoma and breast cancer during sentinel node biopsy (SNB). However, there are still controversies regarding the clinical significance of MI in a variety of tumors including gastric cancer even though it was favored as a significant prognostic factor[2].

MI was considered as a state of dormancy, showing a balance between proliferation and apoptosis without vascular formation, but causing the recurrence after a prolonged period[3,4]. This hypothesis was evident in animal models and human melanoma and breast cancer[5-7]. However, such biologic information on gastric cancer is very limited with data on proliferative activity only[8,9].

Even though SNB is now performed as a practice for limited lymphadenectomy in melanoma and breast cancer, it has not yet been applied to gastric cancer due to unsatisfactory and heterogeneous sensitivity between practicing surgeons with currently available techniques[10]. However, a recently presented prospective multicenter feasibility trial of SNB in gastric cancer showed optimistic results[11]. A single center's observational study after applying SNB in early gastric cancer (EGC) also showed promising results in regard to short and long term results[12]. Based on these results, multicenter phase Ⅲ trial is now planning and quality control studies for it is now underway[13,14]. One of the controversies of SNB application in EGC is the decision of whether radical gastrectomy with lymphadenectomy should be done after detection of MI in the SN[15]. In breast cancer, this issue was confirmed by clinical trials that no further surgical treatment is needed in the case of MI in SNs[16,17]. Applying this approach to gastric cancer is controversial and thus investigation on the clinical significance of MI in EGC should be performed before commencing clinical practice.

The aim of this study was to evaluate the biopathologic features and clinical significance of nodal MI in EGC patients and assess the surgical strategy in these patients during application of SNB.

MATERIALS AND METHODS

Patients and eligibility

Gastrectomy with lymphadenectomy of D1 + β or more was performed in 1022 EGC patients except for cases with an absolute indication of endoscopic resection from March 2001 to December 2005 at the Korean National Cancer Center according to the Japanese guidelines[18]. The final pathology was pT1N0 in 896 (87.7%), pT1N1 in 107 (10.5%), pT1N2 in 16 (1.6%), and pT1N3 in 3 (0.3%) according to the 6th UICC/AJCC staging system[1]. For clinical similarity of metastatic SN, patients with pT1N1 were enrolled in the study. However, tissues of nodal metastasis and primary tumor were available only in 90 of 107 pT1N1 EGC patients. The enrolled patients were divided into MI and MA groups by pathologic findings of metastatic nodes according to the 6th UICC/AJCC staging system, and the findings were compared with each other. Adjuvant chemotherapy of 5-Fluorouracil (5-FU) based regimen was performed in node-positive patients with agreement. The mean follow up period of these 90 patients was 87.6 ± 26.2 mo. Patient recruitment and sample collections were performed according to the study protocol approved by the Institutional Review Board, and informed consent was obtained from all patients (NCCNCS-09-231).

Immunohistochemical stain with cytokeratin and Ki-67

The presence of nodal metastasis was confirmed by IHC for cytokeratin and proliferative activity was measured by IHC for Ki-67 according the previous study[19]. Briefly, primary tumor and metastatic lymph nodes were stored in paraffin-embedded block and then tissue sections of 3 μm in thickness were made. The sections were deparaffinized in xylene, rehydrated through a graded series of alcohol, washed in distillated water and heated twice in a microwave oven for 15 min each at 700 W in 10 mmol/L citrate buffer, pH 6.0 or pH 9.0 to retrieve antigen. After this, it was cooled to room temperature (15-30 min). The activity of endogenous peroxidase was blocked by methanol containing 0.3% H_2O_2 for 10 min and then washed with 0.01 mol/L phosphate buffered saline (PBS). After blocking with 1% normal goat serum for 20 min at room temperature in a humidified chamber, the sections were incubated with primary antibody for 1.5 h at room temperature. The following primary antibodies were used: mouse monoclonal anti-human Ki-67 (clone MIB-1, 1:50) and mouse monoclonal anti-human cytokeratin (clone AE1/AE3, 1:100). After washing in PBS, the specimens were incubated with a biotinylated conjugated-HRP polymer Kit (Super picture, invitrogen, Carlsbad, California) for 30 min at room temperature. As the final step, the slides were developed for 10 min with enzyme substrate 3 and 3-diaminobenzidine (DAB) solution (0.001 mol/L DAB, 0.05 mol/L Tris-HCI buffer, pH 7.6, 0.01 mol/L sodium azide, and 0.006% hydrogen peroxidase). The slides were counterstained with hematoxylin solution for 1 min (DAKO, copenhagen, Denmark). After dehydration, the tissue was sealed with a universal mount (Research Genetics, Huntsville, AL). Controls were prepared in the same manner as detailed for the experimental group, except for the incubation process with primary antibody.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Apoptotic activity was determined *in situ* from the paraffin embedded tissue sections by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the DeadEnd™ Colorimetric TUNEL system (Promega, Madison, WI, United States). The specimens were deparaffinized and gradually hydrated, rinsed with cold 1× PBS, and the sections were fixed in 4% paraformaldehyde for 15 min, followed by incubation with proteinase K (20 μg/mL in 10 mmol/L Tris-HCl, pH 8.0) for 20 min. After washing twice, the sections were equilibrated at room temperature for 10 min in equilibration buffer (200 mmol/L potassium cacodylate, 0.2 mmol/L dithiothreitol, 0.25 g/L bovine serum albumin, and 2.5 mmol/L cobalt chloride in 25 mmol/L Tris-HCL, pH 6.6) and then the slides were covered with the terminal deoxynucleotidyl transferase (TdT) enzyme in a TdT labeling reaction mixture (equilibration buffer, biotinylated nucleotide mix, rTdT enzyme = 8:1:1) for 1 h at 37 ℃ in the dark. The tailing reaction was terminated by 2 × standard saline citrate. The sections were washed three times in PBS and then immersed for 10 min in 0.3% H_2O_2 to block endogenous peroxidase at room temperature. After washing, the sections were subsequently incubated with peroxidase-labeled streptavidin for 30 min at room temperature. Peroxidase activity was visualized with a DAB color reaction and the slides were counterstained with Mayers' hematoxylin, dehydrated, and mounted. After mounting, the sections were observed under a microscope. Positive control sections were treated with 1 μg/mL DNase I (Sigma, St. Louis, MO) for 10 min before treatment with TdT buffer. Negative control sections were treated by substituting distilled water for TdT in the reaction mixture.

Pathologic evaluation

Classification of nodal metastasis was done according to the 6th UICC/AJCC staging system[1]. The main tumor burden with a diameter of no greater than 0.2 mm was defined as ITC, greater than 0.2 mm but no greater than 2 mm as MI, and finally, greater than 2 mm as MA of the representative metastatic node. The location and pattern of nodal metastasis were classified according to previous studies on melanoma and gastric cancer[20,21]. The location of nodal tumor was classified as marginal sinus, intermediate, parenchymal or diffuse type. The pattern of nodal tumor was classified as single cluster, multiple clusters, or diffuse type. Proliferative activity measured by Ki-67 reactivity and apoptotic activity measured by TUNEL assay were defined as the percentage of positive tumor cells per 500 observed tumor cells in the most intensively reacted area. If the number of tumor cells was less than 500, the total tumor cell count itself was used as the denominator.

Statistical analysis

Continuous variables were compared using the Student *t* test or Mann-Whitney *U* test according to the sample size of comparing groups. The χ^2 test or Fisher's exact test was used for comparing categorical variables as the above principle. A scattered plot was created with Pearson's correlation coefficient for proliferative and apoptotic activities of primary and metastatic nodal tumors. *P* values were two sided and values of < 0.05 were considered statistically significant. All data were analyzed using SAS version 9 (SAS Institute Inc., Cary, NC) and interpreted by a biostatistics specialist.

RESULTS

Clinicopathologic features of enrolled patients

The incidence of patients with MI and MA was 41.1% and 58.9% with the mean main tumor burden of 0.9 and 4.6 mm, respectively ($P < 0.001$) (Table 1). Japanese N2 station involvement in MI and MA was 10.3% and 20.9%, respectively. The location of nodal tumor in the MA group was mostly at the non marginal sinus and this

Table 1 Clinicopathologic features according to the classification of lymph node metastasis (*n* = 90)

	Micrometastasis (*n* = 37)	Macrometastasis (*n* = 53)	*P* value
Age (yr)	56.9 ± 12.9	59.2 ± 11.1	0.377
Sex			0.665
Male	22 (59.5)	34 (64.2)	
Female	15 (40.5)	19 (35.8)	
Depth of invasion			0.966
Mucosa	5 (13.5)	7 (13.2)	
Submucosa	32 (86.5)	46 (86.8)	
Tumor size (cm)	4.7 ± 2.8	4.5 ± 2.0	0.652
Histology			0.522
Differentiated	18 (48.6)	30 (56.6)	
Undifferentiated	19 (51.4)	23 (43.4)	
Lauren classification			0.162
Intestinal	19 (51.4)	35 (66.0)	
Diffuse, mixed	18 (48.6)	18 (34.0)	
Lymphovascular invasion			0.832
Absent	17 (45.9)	23 (43.4)	
Present	20 (54.1)	30 (56.6)	
Metastatic LNs (*n*)	1.7 ± 1.3	2.2 ± 1.3	0.085
Japanese N2 station involvement[1]			0.338
No	26 (89.7)	34 (79.1)	
Yes	3 (10.3)	9 (20.9)	
Main tumor burden in LN (mm)	0.9 ± 0.5	4.6 ± 4.6	< 0.001
Pattern of metastasis in LNs			0.694
Single cluster	14 (37.8)	18 (34.0)	
Multiple cluster	22 (59.5)	33 (62.3)	
Diffuse	1 (2.7)	2 (3.8)	
Location of metastasis in LNs			< 0.001
Marginal sinus	17 (45.9)	1 (1.9)	
Non marginal sinus	20 (54.1)	52 (98.1)	
Gastric resection			0.423
Open subtotal	27 (73.0)	40 (75.5)	
Open total	2 (5.4)	6 (11.3)	
LADG	8 (21.6)	7 (13.2)	
Lymph node dissection			0.261
D1 + β	15 (40.5)	15 (28.3)	
D2	22 (59.5)	38 (71.7)	
Dissected LNs (*n*)	36.2 ± 11.1	38.6 ± 15.6	0.424
Adjuvant chemotherapy			0.503
No	11 (29.7)	20 (37.7)	
Yes	26 (70.3)	33 (62.3)	
Recurrence			0.041
Absent	37 (100)	47 (88.7)	
Present	0 (0)	6 (11.3)	

[1]Available data only. Data are expressed as absolute numbers (percentage) or mean ± SD. LN: Lymph node; LADG: Laparoscopically assisted distal gastrectomy.

finding was significantly different with the MI group ($P < 0.001$). The pathologic features regarding the primary tumor was not different between the two groups. All the clinical features were not different between the two groups except recurrence which occurred in 6 patients of the MA group ($P < 0.001$).

Biologic features of primary and metastatic nodal tumors

Representative microscopic photos of IHC with cytokeratin, Ki-67 and TUNEL assay are presented in Figure 1. Proliferative and apoptotic activities of MI were significantly decreased than those of MA among the examined tissues (26.7% *vs* 40.5%, $P = 0.004$ and 1.0% *vs* 3.0%, $P < 0.001$, respectively) (Table 2). The proliferative activity of the primary tumor was not different but the apoptotic activity was different between the two groups. There was a significant correlation between proliferative and apoptotic activities in both the primary tumor and nodal metastasis. Furthermore, both the proliferative and apoptotic activities of nodal metastasis were well correlated to those of the primary tumor (Figure 2).

Table 2 Biologic features according to the classification of lymph node metastasis (*n* = 90)

	Micrometastasis	Macrometastasis	*P* value
Ki-67 (primary tumor)			
Examed tissue	33	51	
Positive cell	59.3% ± 24.0%	62.3% ± 20.9%	0.553
TUNEL (primary tumor)			
Examed tissue	30	51	
Positive cell	2.4% ± 1.7%	4.5% ± 4.4%	0.004
Ki-67 (lymph node)			
Examed tissue	33	50	
Positive cell	26.7% ± 18.0%	40.5% ± 24.1%	0.004
TUNEL (lymph node)			
Examed tissue	25	51	
Positive cell	1.0% ± 1.0%	3.0% ± 3.5%	< 0.001

Data are expressed as absolute numbers (percentage) or mean ± SD. TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Recurrence and associated factors

All 6 recurrent cases had MA and lymphovascular invasion (LVI) of the primary tumor. However, patients without recurrence showed 56.0% MA and 52.4% positive LVI. These factors were statistically significant for recurrence ($P = 0.041$, $P = 0.032$, respectively) (Table 3). Biologic features of proliferative and apoptotic activities in the primary tumor and nodal metastasis were not significant. The details of recurrent patients are shown in Table 4. The sites of recurrence were locoregional, hematogenous and peritoneal as well known patterns of gastric cancer. The number of harvested lymph nodes was only 14 even though D1 + β lymphadectomy was done in case 3.

DISCUSSION

The screening program of gastric cancer for early detection is well established in Asian countries, especially in South Korea and Japan[22,23]. As the proportion of EGC has increased, the biopathological and clinical features of nodal MI in EGC patients have gained attention due to the development of minimally invasive surgery, such as endoscopic submucosal dissection, SNB oriented tailored approach and laparoscopic surgery, in these patients[13,24]. Even though SNB in EGC is not routinely practiced, it is controversial whether lymphadenectomy of D1 + β or more should be performed if MI is dete-

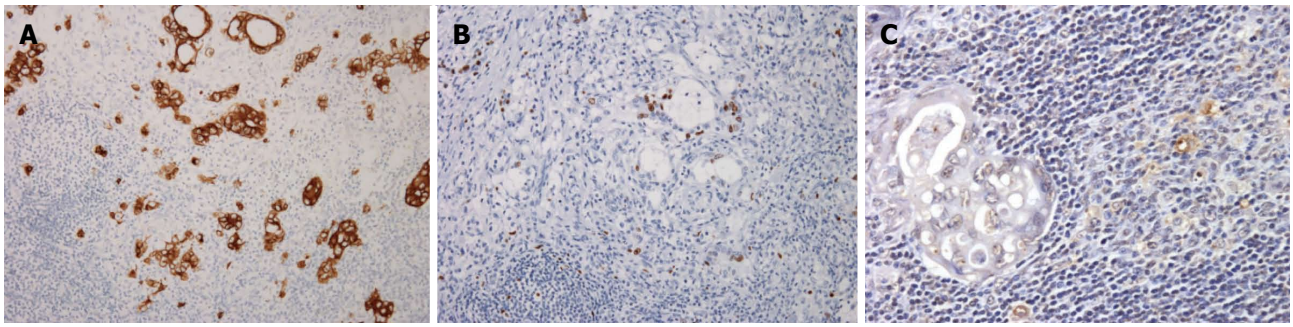

Figure 1 Representative microscopic photos of immunohistochemistry with cytokeratin (A), Ki-67 (B) and terminal deoxynucleotidyl transferase dUTP nick end labeling assay (C).

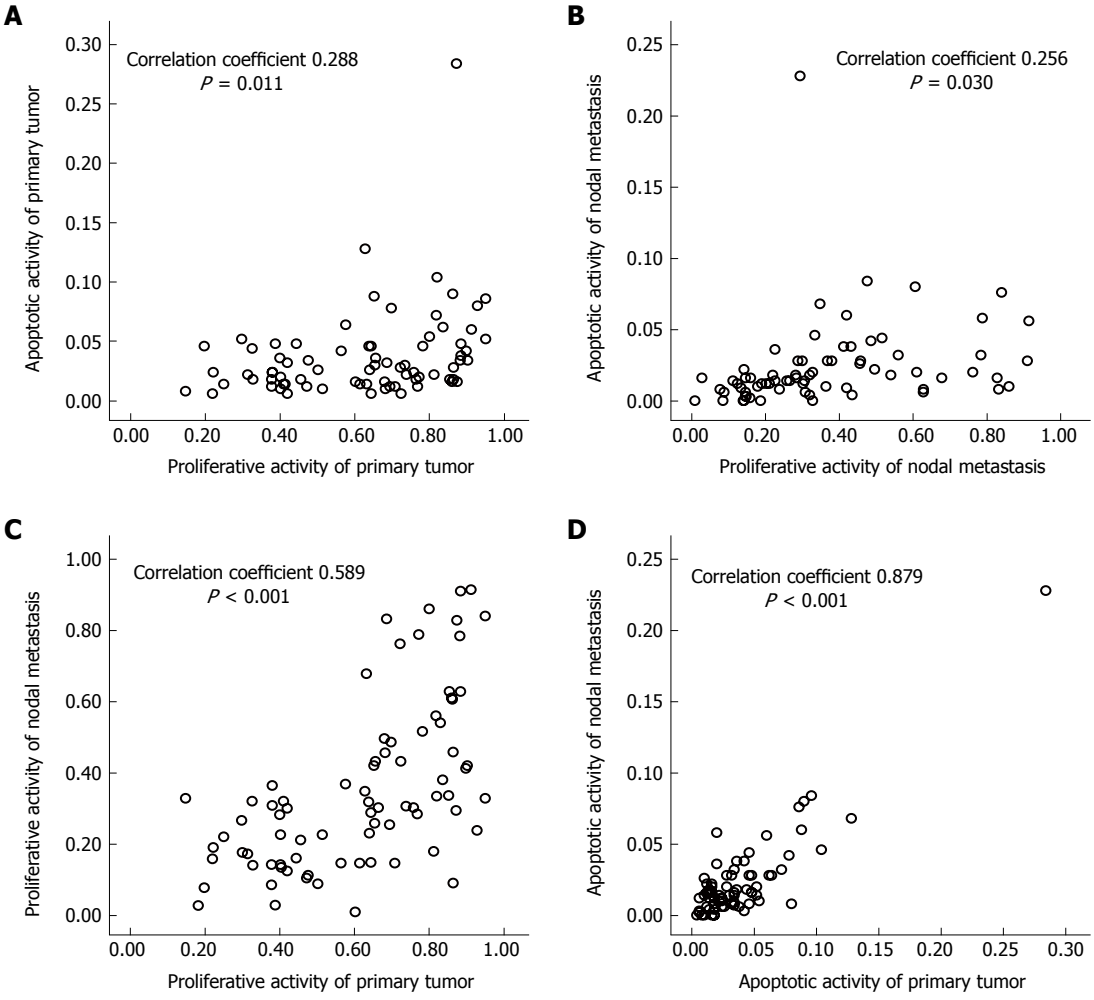

Figure 2 Correlation of proliferative and apoptotic activities in the primary tumor (A), nodal metastasis (B) and with each other (C, D).

cted in SNs[15]. Most nodal MIs in EGC have been studied by comparing its prognostic significance in pN0 patients, and the results are still controversial[15]. However, the present study compared nodal MIs with MAs in pN1 patients to assess the therapeutic strategy. In the present study, we revealed that the nodal MI of EGC has a relatively high proliferative activity and an equivalent apoptotic activity compared to other cancers. Moreover, about 10% of nodal MIs were located at Japanese N2 station. Uncleared lymph nodes at Japanese N2 station in MI, patients may progress to MA and recurrence because most of SNs were located along Japanese N1 station.

The fate of MI in the lymph node is controversial as to whether they will progress to overt metastasis or regress spontaneously by the human immune system. Several animal studies have been reported concerning this issue but direct human evidence is scanty[25,26]. The biology of MI

Table 3 Recurrence and associated factors (*n* = 90)

	No recurred (*n* = 84)	Recurred (*n* = 6)	*P* value
Age (yr)	57.8 ± 12.0	64.8 ± 7.4	0.159
Sex			1.000
Male	52 (61.9)	4 (66.7)	
Female	32 (38.1)	2 (33.3)	
Depth of invasion			1.000
Mucosa	12 (14.3)	0 (0)	
Submucosa	72 (85.7)	6 (100)	
Tumor size (cm)	4.7 ± 2.5	3.7 ± 1.27	0.321
Histology			0.681
Differentiated	44 (52.4)	4 (66.7)	
Undifferentiated	40 (47.5)	2 (33.3)	
Lauren			0.396
Intestinal	49 (58.3)	5 (83.3)	
Diffuse, mixed	35 (41.7)	1 (16.7)	
Lymphovascular invasion			0.032
Absent	40 (47.6)	0 (0)	
Present	44 (52.4)	6 (100)	
Metastatic LNs (*n*)	2.0 ± 1.3	2.2 ± 1.2	0.816
Classification of nodal metastasis			0.041
Micrometastasis	37 (44.0)	0 (0)	
Macrometastasis	47 (56.0)	6 (100)	
Japanese N2 station involvement[1]			0.127
No	58 (85.3)	2 (50.0)	
Yes	10 (14.7)	2 (50.0)	
Main tumor burden in LN (mm)	3.0 ± 4.1	4.0 ± 1.5	0.594
Pattern of metastasis in LNs			0.450
Single cluster	28 (33.3)	4 (66.7)	
Multiple cluster	54 (64.3)	1 (16.7)	
Diffuse	2 (2.4)	1 (16.7)	
Location of metastasis in LNs			0.090
Marginal sinus	31 (36.9)	0 (0)	
Non marginal sinus	53 (63.1)	6 (100)	
Gastric resection			1.000
Open subtotal	63 (75.0)	4 (66.7)	
Open total	7 (8.3)	1 (16.7)	
LADG	14 (16.7)	1 (16.7)	
Lymph node dissection			1.000
D1 + β	28 (33.3)	2 (33.3)	
D2	56 (66.7)	4 (66.7)	
Dissected LNs (*n*)	37.4 ± 13.8	41.0 ± 16.4	0.538
Adjuvant chemotherapy			0.660
No	30 (35.7)	1 (16.7)	
Yes	54 (64.3)	5 (83.3)	
Ki-67 (primary tumor)[1]			
Positive cell	61.3% ± 22.1%	58.3% ± 24.6%	0.748
TUNEL (primary tumor)[1]			
Positive cell	3.6% ± 3.9%	4.6% ± 2.2%	0.552
Ki-67 (lymph node)[1]			
Positive cell	35.0% ± 23.5%	35.0% ± 10.8%	0.996
TUNEL (lymph node)[1]			
Positive cell	2.3% ± 3.2%	2.8% ± 1.1%	0.720

[1]Available data only. Data are expressed as absolute numbers (percentage) or mean ± SD. LN: Lymph node; LADG: Laparoscopically assisted distal gastrectomy; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

in melanoma and breast cancer was interpreted as a concept of being in a balanced dormant state between proliferation and apoptosis before tumor vascularization[3,4]. Reported proliferative and apoptotic activities were 2.4%-12% and 0.2%-0.7% in melanoma and breast cancer, respectively[5-7]. However, the present study showed inconsistent results with melanoma or breast cancer due to high proliferative activity and equivalent apoptotic activity of nodal MI in EGC. The more aggressive biological nature of nodal MI in gastric cancer is indirectly reflected as the survival difference between various cancer types[27]. Most of recurrence in gastric cancer occur within 2-3 postoperative years representing the difference with hypothesis of dormancy. The correlation of proliferative and apoptotic activities in the primary tumor and nodal metastasis also indirectly represents the different biology of gastric cancer with melanoma and breast cancer. The meaning of apoptosis does not only include cell loss but also represents proliferative activity[28,29].

Most studies concerning proliferative and apoptotic activities in gastric cancer were performed in primary tumors rather than in nodal metastasis. Data regarding the biopathologic findings of nodal metastasis are very few in gastric cancer. Yonemura *et al*[8] reported that the proliferative activity was 46.6% in ITC of EGC and concluded that ITC has a poor prognosis. Yanagita *et al*[9] reported that the proliferative activity was 29% in ITC, 92% in MI, and 96% with MA and concluded that ITC and MI should be removed during SNB. They used IHC with anti Ki-67 antibody, as was the case this study, but they did not measure apoptotic activity in nodal metastasis. Apoptotic activity should be measured to estimate the fate of nodal MI combined with the proliferative activity. Variability of proliferative activity measured by Ki-67 in nodal metastasis between these studies and our study might be from several issues such as different handling techniques of tissue samples, subjective nature of IHC and technical diversity[30]. However, the common finding of all of these studies is that a significant proliferative activity is present in nodal MI, even in ITC.

Previous studies with melanoma reported that the tumor burden in SNs is well correlated with the involvement of non-SN and survival[31-33]. The Rotterdam criteria simply measures SN tumor burden by the maximum diameter (in any direction) of the largest lesion. In this study, the main tumor burden in EGC had no clinical significance in terms of recurrence unlike melanoma. However, recurrence was observed only in the MA group. Another important factor for recurrence was determined as LVI in this study. LVI is a well known prognostic factor in gastric cancer[34,35]. Recurrence was not observed in MI group probably because the enrolled patients already received lymphadenectomy of D1 + β or more and this finding offer the indirect suggestion about the surgical strategy when we identified the MI in SN.

Other important factor predicting non SN involvement is the location of metastasis in SNs[20,21]. The location of MI in the parenchyma of SN is significantly related with non SN involvement in melanoma and EGC studies. In this study, a similar finding of non SN involvement could not be proven but the fact that MA had less marginal sinus location than MI indirectly implies disease

Table 4 Details of recurrent cases with pT1N1 in gastric cancer

Case	Age (yr)	Sex	T depth	T size	Histology	Lauren	LVI	Number of metastatic LNs	Size of metastasis (mm)	Pattern	Location	Tumor burden (mm)	Proportion	Extent of LND	Number of dissected LNs	Site of recurrence
1	62	M	Sm3	2.3	Undiff	Diffuse	Present	2	2.3	Single cluster	Parenchyme	2.5	40.0%	D1 + β	31	N3 LN
2	62	M	Sm3	3.0	Diff	Intestinal	Present	2	3.1	Single cluster	Parenchyme	3.88	26.0%	D2	59	Liver
3	72	M	Sm3	3.0	Diff	Intestinal	Present	1	3.6	Diffuse	Diffuse	9.62	65.0%	D1 + β	14	Peritoneum
4	59	M	Sm3	3.0	Diff	Intestinal	Present	4	5.5	Single cluster	Parenchyme	10.8	45.0%	D2	45	Liver, lung
5	76	F	Sm2	5.0	Diff	Intestinal	Present	1	6.0	Single cluster	Parenchyme	6.0	67.0%	D2	54	Liver
6	58	F	Sm3	5.5	Undiff	Intestinal	Present	3	3.2	Multiple cluster	Parenchyme	7.2	87.0%	D2	43	PALN

LVI: Lymphovascular invasion; LN: Lymph node; LND: Lymph node dissection; PALN: Para-aortic lymph node; Diff: Differentiate; Undiff: Undifferentiate.

progression in the lymph node from MI to MA.

For the evaluation of biopathologic and clinical significance of nodal MI and assessment of surgical strategy during SNB, we should have used tissues and information of patients who experienced SNB with EGC at our institution[36]. However, obtaining available tissues from patients for SNB was very limited in our study. Thus, as a second choice we used tissue of pT1N1 patients that simulated the positive SNB results. Therefore, the interpretation of the results of this study has some limitations.

In conclusion, nodal MI in EGC patients has a relatively high proliferative activity and an equivalent apoptotic activity compared to other cancers. Also, not a few patients had Japanese N2 station MI involvement. Therefore, if MI is identified during SNB in EGC, lymphadenectomy of D1 + β or more may be necessary to prevent recurrence by clearing MI involving Japanese N2 station.

COMMENTS

Background

Nodal metastasis is the one of the important prognostic factors along with the depth of invasion of the primary tumor and distant metastasis in solid cancers. However, there are still controversies regarding the clinical significance of micrometastasis (MI) in a variety of tumors including gastric cancer. Surgical strategy in early gastric cancer (EGC) with nodal MI is controversial during the sentinel node biopsy due to the lack of biopathologic and clinical data.

Research frontiers

The current study aimed to evaluate the biopathologic features and clinical significance of nodal MI in EGC patients and assess the surgical strategy in these patients during application of sentinel node biopsy.

Innovations and breakthroughs

Nodal micrometastasis in EGC has a relatively high proliferative and an equivalent apoptotic activities compared to other cancers such as breast cancer or melanoma. The incidence of Japanese N2 station micrometastasis involvement is about 10%.

Applications

Lymphadenectomy of D1 + β or more might be necessary if micrometastasis is identified during sentinel node biopsy in EGC.

Terminology

The main tumor burden with a diameter of no greater than 0.2 mm was defined as isolated tumor cell, greater than 0.2 mm but no greater than 2 mm as MI, and finally, greater than 2 mm as macrometastasis of the representative metastatic node according to the 6th International Union Against Cancer/American Joint Committee on Cancer staging system.

Peer review

In this retrospective study, the authors assessed quite a large number of gastrectomy cases to identify factors associated with nodal metastasis for early gastric cancer. They classified nodal metastasis into 2 groups; micrometastasis and macrometastasis based on the main tumor burden. They assessed proliferative and apoptotic activities of the primary tumor by immunohistochemical staining. From their results, nodal micrometastasis showed a relatively high proliferative activity and an equivalent apoptotic activity. They concluded that extensive lymphadenectomy might be necessary in patients with micrometastasis to prevent recurrence. This is a carefully done study and the findings are of considerable interest.

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SYSTEMATIC REVIEWS

Accurate definition and management of idiopathic sclerosing encapsulating peritonitis

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Abstract

AIM: To review the literature on idiopathic sclerosing encapsulating peritonitis (SEP), also known as abdominal cocoon syndrome.

METHODS: The PubMed, MEDLINE, Google Scholar, and Google databases were searched using specific key words to identify articles related to idiopathic SEP. These key words were "sclerosing encapsulating peritonitis," "idiopathic sclerosing encapsulating peritonitis," "abdominal cocoon," and "abdominal cocoon syndrome." The search included letters to the editor, case reports, review articles, original articles, and meeting presentations published in the English-language literature from January 2000 to May 2014. Articles or abstracts containing adequate information about age, sex, symptom duration, initial diagnosis, radiological tools, and surgical approaches were included in the study. Papers with missing or inadequate data were excluded.

RESULTS: The literature search yielded 73 articles on idiopathic (primary) SEP published in 23 countries. The four countries that published the greatest number of articles were India (*n* = 21), Turkey (*n* = 14), China (*n* = 8) and Nigeria (*n* = 3). The four countries that reported the greatest number of cases were China (*n* = 104; 53.88%), India (*n* = 35; 18.13%), Turkey (*n* = 17; 8.80%) and Nigeria (*n* = 5; 2.59%). The present study included 193 patients. Data on age could be obtained for 184 patients (range: 7-87 years; mean ± SD, 34.7 ± 19.2 years), but were unavailable for nine patients. Of the 184 patients, 122 were male and 62 were female; sex data could not be accessed in the remaining nine patients. Of the 149 patients whose preoperative diagnosis information could be obtained, 65 (43.6%) underwent operations for abdominal cocoon, while the majority of the remaining patients underwent operations for a presumed diagnosis of intestinal obstruction and/or abdominal mass. Management information could be retrieved for 115 patients. Of these, 68 underwent excision + adhesiolysis (one laparoscopic); 24 underwent prophylactic appendectomy in addition to excision + adhesiolysis. Twenty patients underwent various resection and repair techniques along with excision + adhesiolysis. The remaining three patients were managed with antituberculosis therapy (*n* = 2) and immunosuppressive therapy (*n* = 1).

CONCLUSION: Idiopathic SEP is a rare disorder characterized by frequently recurring bouts of intestinal obstruction. Surgical therapy is the gold standard

management strategy.

Key words: Primary; Idiopathic; Intestinal obstruction; Sclerosisis encapsulation peritonitis; Abdominal cocoon syndrome

Core tip: Idiopathic sclerosing encapsulating peritonitis (SEP) is a clinical entity characterized by partial or complete encasement of the small intestines by a thick fibrocollagenous membrane. While some patients with idiopathic SEP are asymptomatic, the majority of affected individuals develop acute, subacute or chronic attacks of gastrointestinal obstruction. Preoperative diagnosis of the disease is quite difficult, and many cases are diagnosed intraoperatively. Nonetheless, recent technological advances in imaging modalities, particularly computed tomography, have made preoperative diagnosis of SEP possible. Surgery remains the best management option for patients with severe signs of intestinal obstruction.

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INTRODUCTION

Sclerosing encapsulating peritonitis (SEP) is a chronic inflammatory process in which the small intestines are encased by a dense fibrocollagenous membrane[1-32]. SEP was first defined nearly 100 years ago, at which time it was termed "peritonitis chronica fibrosa incapsulata"[1,6,7]. The disorder is divided into primary (idiopathic) and secondary forms according to the underlying etiological cause[1-5]. The primary form was termed "abdominal cocoon syndrome" by Foo in 1978[1]. The clinical signs and symptoms of SEP vary with the severity and duration of the disease, underlying causes, and affected person's immunological status. SEP most commonly manifests as recurrent acute, subacute, or chronic episodes of intestinal obstruction[2,4]. However, some cases may also manifest with more uncommon, but life-threatening, complications including enterocutaneous fistula, small intestinal necrosis, and malnutrition. Preoperative diagnosis of SEP is quite difficult, and many cases are diagnosed intraoperatively[4,6]. Fortunately, preoperative diagnosis of SEP has become possible with recent technological advances in imaging modalities, particularly computed tomography (CT)[1,5-11]. Surgery remains the most effective management option for SEP[4], although controversy surrounds the indications, optimal timing, and mode of surgical operation. This is because surgical outcomes are far from satisfactory, and some patients may develop postoperative small intestinal obstruction and new adhesions[4]. The present study reviews and discusses the previously published articles on SEP.

MATERIALS AND METHODS

We reviewed nearly 200 previously published articles on SEP. A serious contradiction was present between selection and classification of cases, because many authors used the term "abdominal cocoon" while actually describing cases of secondary SEP. We therefore aimed to resolve this conflict by establishing a proper definition and classification of SEP before starting the literature review. We divided SEP into primary (idiopathic; abdominal cocoon syndrome) and secondary forms. Patients with no factors explaining SEP after various examinations (history taking, blood tests, radiological imaging, and histopathological tests) performed during the preoperative, perioperative, or postoperative periods were determined to have primary SEP (idiopathic, abdominal cocoon). Patients with SEP that developed as a result of various conditions, including abdominal surgery, abdominal tuberculosis, peritoneal dialysis (PD), ventriculoperitoneal or peritoneovenous shunts, liver transplantation, recurrent peritonitis, beta-blocker treatment (practolol or propranolol), intraperitoneal chemotherapy, intraperitoneal povidone-iodine use, liver cirrhosis, gastrointestinal malignancy, fibrogenic foreign material, systemic lupus erythematosus, or parasitic infection (sometimes leading to granulomatous peritonitis) were determined to have secondary SEP. The main objective of the present study was to perform a brief review of the literature to identify studies on primary SEP (idiopathic; cocoon syndrome) published from January 2000 to May 2014. To achieve this aim, we scanned the PubMed, MEDLINE, Google Scholar, and Google databases for the key words "sclerosing encapsulating peritonitis," "idiopathic sclerosing encapsulating peritonitis," "abdominal cocoon," and "abdominal cocoon syndrome" entered alone or in various combinations. Only articles published in English were included in the scanning process. Cases that met the diagnostic criteria for idiopathic SEP (abdominal cocoon) were included in the review, while cases with features of secondary SEP were excluded. The corresponding authors of some papers were e-mailed several times regarding necessary information about their articles. However, we received no effective responses from the authors of the two largest studies. We created a table with useful information about the reviewed articles, including publication year, country, number of cases, patient age, sex, history, white blood cell count, surgical approach, complications, follow-up duration and other ancillary information.

RESULTS

A literature review using the above mentioned inclusion criteria revealed 73 articles on idiopathic (primary) SEP

Table 1 Distribution of articles and number of cases with idiopathic sclerosing encapsulating peritonitis according to countries *n* (%)

Countries	Published articles	Published cases
China	8 (10.95)	104 (53.88)
India	21 (28.76)	35 (18.13)
Turkey	14 (19.17)	17 (8.80)
Nigeria	3 (4.10)	5 (2.59)
Taiwan	2 (2.74)	2 (1.03)
Pakistan	2 (2.74)	2 (1.03)
Qatar	2 (2.74)	3 (1.55)
Saudi Arabia	2 (2.74)	2 (1.03)
Israel	2 (2.74)	3 (1.55)
Iran	2 (2.74)	2 (1.03)
Nepal	2 (2.74)	2 (1.03)
Brazil	2 (2.74)	3 (1.55)
Italy	1 (1.37)	1 (0.51)
United States	1 (1.37)	2 (1.03)
South Korea	1 (1.37)	2 (1.03)
Senegal	1 (1.37)	1 (0.51)
Iraq	1 (1.37)	1 (0.51)
Belgium	1 (1.37)	1 (0.51)
Bangladesh	1 (1.37)	1 (0.51)
Kuwait	1 (1.37)	1 (0.51)
Malaysia	1 (1.37)	1 (0.51)
New Zealand	1 (1.37)	1 (0.51)
Greece	1 (1.37)	1 (0.51)

China has reported the greatest number of cases, while India has published the greatest number of articles.

from 23 countries[2-10,12-31,33-76]. The four countries with the highest numbers of published articles were India (*n* = 21; 28.76%), Turkey (*n* = 14; 19.17%), China (*n* = 8; 10.95%) and Nigeria (*n* = 3; 4.10%). The four countries reporting the highest number of cases were China (*n* = 104; 53.88%), India (*n* = 35; 18.13%), Turkey (*n* = 17; 8.80%) and Nigeria (*n* = 5; 2.59%). Other data related to the article distribution among countries are presented in Table 1. In total, 193 patients were included in this study. Their ages ranged from 7 to 87 years (mean ± SD, 34.7 ± 19.2 years) among 184 patients; this information was unavailable for the remaining 9 patients. Of the 184 patients, 122 were male and 62 were female; no sex data were available for the remaining 9 patients. The symptom duration ranged from 8 h to 210 mo among 174 patients; this information was unavailable for the remaining 19 patients. Of 149 patients with available data on preoperative diagnosis, 65 (43.6%) underwent operations for a presumed diagnosis of abdominal cocoon syndrome, while the majority of the remaining patients underwent operations for an initial diagnosis of intestinal obstruction and/or abdominal mass. Patient management data were available in 115 patients; 68 underwent excision + adhesiolysis, and 24 underwent prophylactic appendectomy in addition to excision + adhesiolysis. Twenty patients underwent various resection and anastomosis techniques in addition to excision + adhesiolysis. Two patients commenced antituberculous therapy without antecedent surgical therapy. Those patients had no signs or symptoms pertaining to tuberculosis. One patient was administered with steroids and immunosuppressive therapy. The demographic and clinical data of the 193 patients included in the present study are summarized in Table 2. Two studies were published from the same institution and used the medical data of the same patient; despite meeting the inclusion criteria for this review, one of these studies was excluded[60,77].

DISCUSSION

Definitions and historical background

The definition of SEP is associated with confusion and lack of information. The concepts of primary and secondary SEP are erroneously used interchangeably in many previously published articles on SEP[11,32]. Thus, we aimed to emphasize the correct use of the definitions of peritoneal encapsulation (PE), abdominal cocoon, idiopathic SEP, and secondary SEP in the present review.

PE was first described by Cleland in 1868[32]. It is a developmental anomaly characterized by the congenital presence of an accessory peritoneal membrane, which is believed to be derived from the yolk sac peritoneum in the early stages of fetal life[10,15,29,32]. This peritoneal membrane is classically found between the mesocolon and omentum, and most of the small intestines lie posterior to this membrane[21,27,39,48,75]. In other words, PE is an anatomical anomaly unrelated to any inflammatory process. PE is typically asymptomatic and incidentally detected during laparotomy performed for other indications[29,32,62,73]. In one patient, we observed anatomical features similar to those of PE during laparotomy performed to treat a gunshot injury (Figure 1).

Unlike PE, SEP is an acquired condition resulting from peritoneal inflammation that may be triggered by various factors[32,38]. While the accessory peritoneal membrane is covered by mesothelium in patients with PE, the membrane that encases the intestines in patients with SEP has a dull, fibrous structure that includes inflammatory cells[33,38,39]. SEP is a clinical entity characterized by partial or complete encasement of the small intestines by a thick fibrocollagenous membrane (Figure 2)[1,4,6,10,17,24]. This membrane often encapsulates the small intestines, but it sometimes also encases other intraperitoneal organs, such as the stomach, liver, and colon[1,6,8,23,55]. This clinical entity was first defined in 1907 by Owtschinnikow, who described encasement of the intestines by a fibrocollagenous membrane[1,50,55]. Considering the morphological and histological properties of the membrane encasing the intestines, Owtschinnikow termed this condition "peritonitis chronica fibrosa incapsulata"[1,16,17,27]. Historically, SEP was classified as primary (idiopathic) or secondary, depending on its underlying cause and the pathogenetic properties of the fibrocollagenous membrane[1,23,42,49]. The idiopathic form of SEP has also been termed "abdominal cocoon syndrome," a term that was first used by Foo in 1978[1]. Abdominal cocoon is categorized into three types

Table 2 Demographic and clinical characteristics of 193 patients with idiopathic sclerosing encapsulating peritonitis

Ref.	Year	Country	Case number	Age (yr)	Sex	Duration symptom	Preoperative diagnosis	Radiologic tools	Surgical approach
Rasihashemi *et al*[2]	2014	Iran	1	25	M	2 mo	Int Obst	X-ray + Barium + CT	E + A
Nanwadekar *et al*[3]	2014	India	1	17	F	4 d	Int Obst	X-ray + US + Endosc.	E + A
Li *et al*[4]	2014	China	65	39 (14-79)	M: 57 F: 8	3.9 ± 6.7 yr	ACS: 31 NS: 34	NS	NS
Jovani *et al*[5]	2014	Italy	1	44	M	60 mo	ACS	US + CT + MR	NS
Akbulut *et al*[6]	2014	Turkey	1	87	M	3 mo	Int Obst + perforation	X-ray + US	E + A + resection + ileostomy
Sreevathsa *et al*[7]	2013	India	3	43	M	12 mo	ACS	X-ray + CT	E + A
				13	F	12 mo	Int Obst	X-ray	Ileocecal resection
				14	F	6 mo	Int Obst (Subacute)	X-ray	Ileocecal resection
Singh *et al*[8]	2013	India	9	NS	NS	NS	NS: 9	NS	NS
Shah *et al*[9]	2013	India	1	14	F	6 mo	ACS	Barium + CT	E + A
Serter *et al*[10]	2013	Turkey	2	32	M	2 d	Int Hernia	X-Ray + CT	E + A
				49	M	1 wk	ACS	CT	E + A
Rahmati *et al*[12]	2013	Iran	1	50	M	3 mo	ACS	US + CT + Endosc.	E + A
Patel *et al*[13]	2013	India	1	45	M	6 mo	Int Obst	X-ray + CT	E + A + ileal resection
Ozkan *et al*[14]	2013	Turkey	1	48	M	1 wk	ACS	X-ray + CT	E + A
Hu *et al*[15]	2013	China	1	29	F	Asympt.	Infertility	US	E + A + suturing (iatrogenic ileal injury)
Gupta *et al*[16]	2013	India	1	40	M	NS	ACS	X-ray + US + CT	E + A
Gadhire *et al*[17]	2013	India	1	35	M	1 mo	ACS	X-ray + US + CT	E + A
Awe[18]	2013	Nigeria	1	18	F	3 d	Int Obst	X-ray	E + A
Al Thani *et al*[19]	2013	Qatar	1	41	M	7 mo	Int Obst (subacute)	CT	E + A
Thakur *et al*[20]	2012	India	1	14	F	6 mo	Abd Mass	US	E + A
Taylor *et al*[21]	2012	N Zealand	1	42	M	3 d	Int Obst	X-ray + CT	E + A + appendectomy
Solak *et al*[22]	2012	Turkey	1	58	M	24 mo	ACS (previously operated)	X-ray + US + CT	Steroid + mycophenolate mofetil
Shakya *et al*[23]	2012	Nepal	1	20	M	12 mo	Int Obst	X-ray	E + A + Ileostomy (iatrogenic ileal injury)
Ndiaye *et al*[24]	2012	Senegal	1	15	F	2 mo	ACS	Barium + CT	E + A + Suturing (iatrogenic ileal injury)
Meshikhes *et al*[25]	2012	Saudi Arabia	1	45	M	6 mo	Int Obst + Abd mass	CT	E + A + appendecectomy
Malik *et al*[26]	2012	Pakistan	1	24	F	60 mo	Int Obst	X-ray	E + A
Kumar *et al*[27]	2012	India	2	18	F	24 mo	ACS ?	Barium + US + CT	Antitubercular therapy
				14	F	NS	ACS ?	CT + US	Antitubercular therapy
Kayastha *et al*[28]	2012	Pakistan	1	13	F	2 mo	Acute appendicitis	US	E + A
Kaur *et al*[29]	2012	India	2	43	M	180 mo	ACS	X-ray + US + CT	E + A
				17	F	4 mo	ACS	X-ray + US + CT	E + A
Araujo Filho *et al*[30]	2012	Brazil	1	36	M	10 d	ACS	US + CT	E + A
Chatura *et al*[31]	2012	India	1	14	F	NS	Int Obst + Abd mass	US	E + A + ileocolectomy
Yeniay *et al*[33]	2011	Turkey	2	26	F	2 d	Int Obst	X-ray + CT	E + A
				71	M	3 mo	Int Obst	X-ray + CT	E + A
Kirshtein *et al*[34]	2011	Israel	1	82	M	4 d	Int Obst	X-Ray + gastrografin	E + A
Jayant *et al*[35]	2011	India	1	16	F	NS	Int Obst	CT	E + A
Gupta *et al*[36]	2011	Nepal	1	42	M	4 mo	ACS	X-ray + US + CT	E + A
Ertem *et al*[37]	2011	Turkey	1	29	M	2 d	Int Obst	X-ray + US + CT	E + A - laparoscopic
Da Luz *et al*[38]	2011	Brazil	2	30	M	NS	Int Obst + Int Hernia	X-ray + barium	E + A + laparostomy
				32	M	6 mo	Int Obst + Chron?	X-ray + barium	E + A
Bassiouny *et al*[39]	2011	Qatar	2	7	M	48 mo	Int Obst + Abd mass	X-ray	E + A
				12	F	48 mo	Int Obst	X-ray + US	E + A
Wang *et al*[40]	2010	China	1	48	M	3 mo	ACS	CT	E + A + appendectomy
Tombak *et al*[41]	2011	Turkey	1	36	M	1 mo	ACS	CT	E + A
Naik *et al*[42]	2010	India	1	70	M	48 mo	Int Obst	X-ray + US + CT + Endosc.	E + A

Lee *et al*[43]	2010	Taiwan	1	57	F		ACS	X-ray + US + CT	E + A
Gurleyik *et al*[44]	2010	Turkey	1	30	M	36 mo	Int Obst	X-ray + US + CT	E + A
Al Saied *et al*[45]	2010	Saudi Arabia	1	24	M	36 mo	ACS	X-ray + CT	E + A
Yang *et al*[46]	2009	China	1	43	M	NS	NS	X-ray + Endosc.	Resection (?)
Yang *et al*[47]	2009	China	6	43.7 (39-48)	M: 4 F: 2	3-60 mo	Int Obst: 5 ACS: 1	X-ray + CT	E + A: 5 E + A + jejunal resection: 1
Wu *et al*[48]	2009	Taiwan	1	80	M	24 mo	Int Obst	X-ray + US + CT	E + A
Wei *et al*[49]	2009	China	24	34 (15-57)	M: 9 F: 15	3 d-216 mo	ACS: 4 Int Obst/mass: 20	X-ay + barium + US + CT	E + A + appendectomy: 17 E + A + enterotomy: 2 E + A + cecofixation: 2 E + A: 3
Tasdelen *et al*[50]	2009	Turkey	1	85	F	3 d	Int Obst + Int Hernia	X-ray + CT	E + A + jejunoileal resection with anastomosis
Reynders *et al*[51]	2009	Belgium	1	40	M	36 mo	Int Obst	X-ray + CT	E + A + Meckel's resection + appendectomy
Mohanty *et al*[52]	2009	India	1	15	F	24 mo	ACS	X-ray + US + CT	E + A
Kumar *et al*[53]	2009	India	3	45	M	24 mo	ACS	X-ray + CT	E + A
				63	M	216 mo	ACS	X-ray + US + CT	E + A
				16	F	10 h	ACS	X-ray + US + CT	E + A
Ibrahim *et al*[54]	2009	Nigeria	1	14	M	72 h	Int Obst	X-ray	E + A + appendectomy
Choudhury *et al*[55]	2009	Bangladesh	1	15	F	12 mo	Appendiceal mass	US	Partial ileocolic resection with anastomosis
Zheng *et al*[56]	2008	China	1	69	M	1 d	ACS	X-ray + US + CT	E + A + ileal resection with anastomosis
Bas *et al*[57]	2008	Turkey	1	42	M	5 mo	Int Obst	X-ray + CT	E + A
Singh *et al*[58]	2008	India	1	38	M	12 mo	Int Obst	X-ray + US	E + A
Xu *et al*[59]	2007	China	5	41	F	4 mo	Int Obst	X-ray + CT + Endosc.	E + A
				49	F	120 mo	Int Obst	X-ray + CT + Endosc.	E + A
				21	M	36 mo	Int Obst	X-ray + CT + Endosc.	E + A
				41	M	1 mo	Int Obst	X-ray + CT + Endosc.	Adhesiolysis + jejunal resection with anastomosis
				36	M	2 wk	Int Obst	X-ray + CT + Endosc.	E + A
Demir *et al*[60]	2007	Turkey	1	38	M	6-7 mo	ACS	CT	E + A
Cai *et al*[61]	2007	United States	2	38	M	2 d	Int Obst	X-ray	E + A
				45	M	8 h	Int Obst	CT	E + A
Basu *et al*[62]	2007	India	1	47	M	3 mo	Abd mass	X-ray + US + barium	E + A
Al-Ibrahim *et al*[63]	2007	Kuwait	1	33	M	1 mo	Int Obst	X-ray + US + CT	E + A
Serafimidis *et al*[64]	2006	Greece	1	56	M	48 mo	Int Obst	X-ray + US + CT + Endosc.	E + A
Rokade *et al*[65]	2006	India	1	26	F	12 mo	ACS (previously operated)	US + CT	E + A
Pillai *et al*[66]	2006	India	1	13	F	NS	ACS	X-ray + US + CT	E + A
Akca *et al*[67]	2006	Turkey	1	57	M	75 d	Int Hernia + mesenteritis	US + CT + Colonosc.	NS
Yucel *et al*[68]	2004	Turkey	2	15	F	NS	Int Obst	X-ray + CT	E + A
				38	M	72 mo	Int Obst	X-ray + CT	E + A
Hur *et al*[69]	2004	South Korea	2	34	F	120 mo	Int Obst	X-ray + barium + US + CT	NS
				47	M	NS	Int Obst	X-ray + barium + CT	NS
Vijayaraghavan *et al*[70]	2003	India	1	12	F	3 mo	ACS + Int Hernia	US	E + A
Ranganathan *et al*[71]	2003	Malaysia	1	25	F	3 mo	Large ovarian mass + ascites	US + CT	E + A
Hasan[72]	2002	Iraq	1	20	F	NS	Acute abdomen	Pregnant patient	E + A
Hamaloglu *et al*[73]	2002	Turkey	1	38	M	12 mo	Int Obst	X-ray + Barium + US	E + A
Okobia *et al*[74]	2001	Nigeria	3	18	F	5 mo	Pelvic collection	US	E + A + appendectomy
				12	F	1 wk	Mesenteric cyst	X-ray + US	E + A + appendectomy
				10	F	2 mo	Ovarian Tm + Burkitt's Tm + uterine mass	X-ray + Urography	E + A + appendectomy

Mordehai *et al*[75]	2001	Israel	2	14	F	1 mo	Int Obst	X-ray + US + CT	E + A
				15	F	6 mo	Int Obst	X-ray + US	E + A
Kumar *et al*[76]	2000	India	1	12	F	24 h	Int Obst	X-ray + US	E + A

CT: Computed tomography; US: Ultrasonography; X-Ray: Plain X-ray abdominal radiography; Endosc: Gastrointestinal endoscopy; Int Obst: Intestinal obstruction; ACS: Abdominal cocoon syndrome; Abd mass: Abdominal mass; Int Hernia: Internal herniation; NS: Non-stated; E + A: Excision + adhesiolysis.

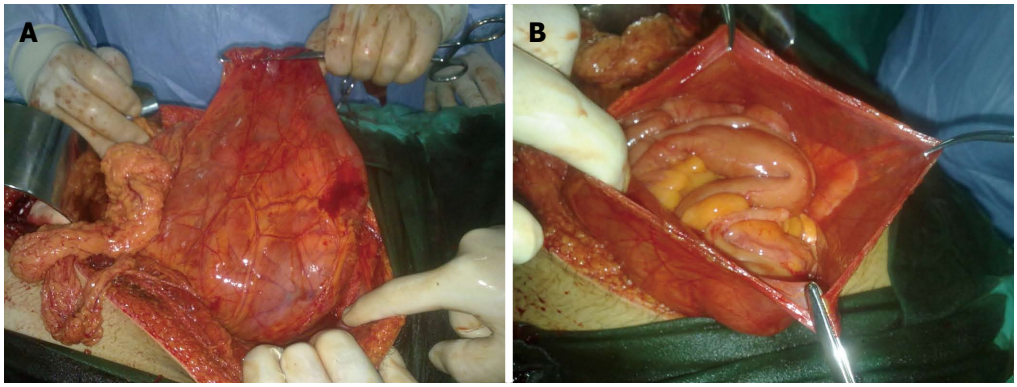

Figure 1 Bowel encased in a membranous sac suggestive of peritoneal encapsulation. A: The overall appearance of the membranous sac is shown. All intestines are localized behind the accessory peritoneal membrane; B: The appearance of the opened membranous sac is shown.

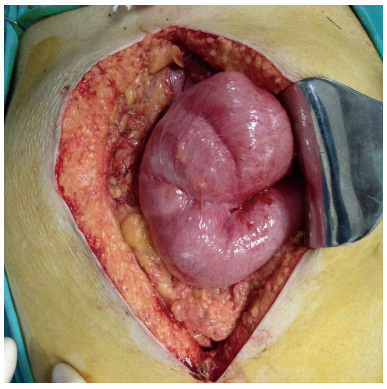

Figure 2 Intraoperative photograph showing the encapsulated small bowel (dense, cocoon-like fibrous membrane).

according to the extent of the encasing membrane that covers the intestine. Encasement of part of the intestine by a fibrocollagenous membrane is called type 1 cocoon syndrome. Complete coverage of the intestine by the membrane is called type 2 abdominal cocoon syndrome. Type 3 cocoon syndrome refers to encasement of the whole intestine, as well as other intra-abdominal organs, such as the appendix, cecum, ascending colon, and ovaries[1,49].

Etiology

SEP is considered to be primary (idiopathic) or secondary, depending on its underlying cause[1-10]. No underlying cause can be demonstrated in primary SEP, although the role of cytokines and fibroblasts in development of peritoneal fibrosis and neoangiogenesis is indisputable[40,58]. Idiopathic SEP classically presents in young adolescent girls in tropical and subtropical countries such as China, Malaysia, Singapore, Pakistan, India, Nigeria, Kenya, Saudi Arabia, and South Africa, although adult cases of idiopathic SEP in temperate zones have also been reported[1,4,7,22,23,58,61]. The present study showed that idiopathic SEP is twice as common in men than in women. Our findings on the geographical distribution of SEP coincide with those in the previously published literature. Indeed, nearly all cases presented herein occurred in tropical or subtropical regions of the world.

Many hypotheses regarding the etiology of idiopathic SEP have been proposed[55,59,64]. Some of these hypotheses involve retrograde menstruation with a superimposed viral infection, retrograde peritonitis *via* the fallopian tubes, and cell-mediated immunological tissue damage secondary to gynecological infection[1,4,7,13,23,28,36,39]. However, SEP also develops in men, premenopausal women, and children, reducing support for these theories[1,4,7,28,61]. In total, 66 of 89 patients included in the largest two studies on idiopathic SEP in the literature to date were male[4,49]. Some authors have argued that the fibrous membrane that encases the intestines is a result of a developmental disorder, citing vascular anomalies and omental hypoplasia as the basis of their hypothesis[1,49,59].

Secondary SEP is more common than idiopathic SEP[22,45,52]. In secondary SEP, a local or systemic factor triggers the inflammatory process in the peritoneum[52]. PD is the most common cause of secondary SEP[1]. In other words, secondary SEP is the leading cause of the most severe complications of PD. This is because once secondary SEP has developed, the ultrafiltration capacity of the peritoneal surface decreases and the risk of intestinal obstruction increases[1]. Studies have shown a direct relationship between prolonged PD and

Table 3 Classification of sclerosing encapsulating peritonitis according to underlying cause

Primary (idiopathic) sclerosing encapsulating peritonitis
Ⅰ Adolescent form
Ⅱ Adult form
Secondary sclerosing encapsulating peritonitis
Ⅰ Systemically induced by
Beta adrenergic blocking agents
Practolol
Timolol
Propanolol
Other drugs
Methotrexate
Protein S deficiency
Exposure to asbestosis
Ⅱ Induced by possible local and/or systemic irritants
Peritoneal dialysis
Abdominal trauma
Abdominal surgery
Liver transplantation
Peritoneovenous shunt
Ventriculoperitoneal shunt
Peritoneal sarcoidosis
Liver cirrhosis
Peritoneal tuberculosis
Sarcoidosis
Familial mediterranean fever
Systemic lupus erythematosus
Gastrointestinal malignancy
Intraperitoneal chemotherapy
Fibrogenic foreign body
Endometriosis
Dermoid cyst rupture
Luteinized ovarian thecomas
Cytomegalovirus peritonitis
Recurrent peritonitis
Granulomatous peritonitis related with parasitic infestation

the development of secondary SEP[1,11]. Considering the number of patients undergoing PD worldwide, the importance of the relationship between PD and secondary SEP needs to be better understood. Abdominal tuberculosis continues to be a major public health issue and an important etiological agent of secondary SEP in underdeveloped countries[8]. Among the less frequent causes of secondary SEP are a history of abdominal surgery, autoimmune disorders, some drugs, peritoneal shunts, and recurrent episodes of peritonitis[1,4,7,10,17,28,32,34]. The classification and potential etiological factors of SEP are listed in detail in Table 3.

Clinical presentation

Idiopathic SEP is an uncommon entity, and a great majority of physicians either never encounter patients with this condition or miss the diagnosis even when they do. Achieving a correct preoperative diagnosis in affected patients is extremely difficult and requires a high index of clinical suspicion[1,4,25,38,51]. Recent advances in radiological modalities have allowed physicians to achieve a correct preoperative diagnosis of SEP in affected patients[59,71,77]. Nevertheless, preoperative diagnosis remains a clinical challenge because most patients with SEP present to emergency departments with signs and symptoms of intestinal obstruction, and many emergency departments lack advanced radiological equipment and adequate staff, and patients with this syndrome usually undergo operations on an urgent basis[38]. In one large case series, 52.3% to 100.0% of admitted patients were diagnosed during surgery and 16.7% to 48.7% were diagnosed during their preoperative examinations[4,8,50]. While some patients with SEP are asymptomatic, most affected individuals develop acute, subacute, or chronic attacks of gastrointestinal obstruction (incomplete or complete); nausea; vomiting; anorexia; appetite loss; weight loss; and malnutrition[1,4,8,10,11,26]. Although rare, a painless, soft abdominal mass can be palpated in some patients[1,4,8,29,30,76]. Additionally, abdominal ascites and distention are detectable in some patients with severe disease. Ascites may be massive enough to induce suspicion of underlying hepatic disease. Primary SEP may be considered in patients presenting with recurrent attacks of abdominal pain who are free of any disease explaining such attacks[1]. Gastrointestinal perforation is quite rare in patients with SEP; of all reported cases of SEP, only two (one secondary to tuberculosis and the other idiopathic) were associated with spontaneous perforation[6].

Diagnostic approaches

The diagnosis of SEP is often made by a combination of the medical history, a high clinical index of suspicion, various biochemical parameters, and radiological findings[18,23,26]. The patient's medical history (tuberculosis, PD, systemic lupus erythematosus, previous abdominal operations, *etc.*) usually provides important clues regarding the etiology of secondary SEP. The most commonly used radiological techniques are abdominal X-rays, small intestinal barium studies, ultrasonography, abdominal CT, and occasionally contrast-enhanced magnetic resonance (MR) imaging[5,6,28,30]. Abdominal X-rays may show diffuse air-fluid levels and dilated small intestinal loops[1,3,29,35]. However, X-ray findings are not specific to idiopathic SEP; rather, they are common to many conditions characterized by intestinal obstruction[22]. In patients with SEP, small intestinal barium studies show the intestinal loops that are accumulated and conglomerated at the center of the abdomen (Figure 3A)[1,9,24,29,76]. This appearance is termed the cauliflower sign or accordion pattern and is a clue for the diagnosis of SEP[9,24,29,64,66,69]. A prolonged transit time may also aid in the diagnosis (Figure 3B)[1,23,24,38]. However, barium studies may not be possible in patients with prominent signs of intestinal obstruction. Abdominal ultrasonography may show dilated bowel segments encased by a dense fibrous membrane[1,44] or free abdominal fluid and a thickened peritoneal layer[22,29,68]. Contrast-enhanced CT is the most helpful imaging modality for the diagnosis of abdominal cocoon[3,29,36]. The characteristic sign on CT is the appearance of small bowel segments that are conglomerated at the

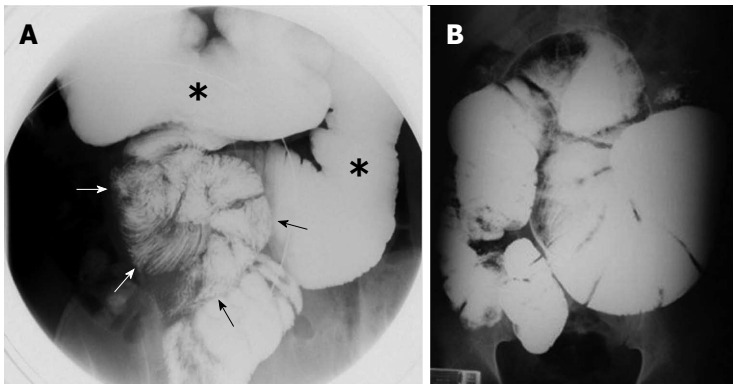

Figure 3 Small bowel transit. Procubitus image with localized compression. Liquid distension of the gastroduodenum (asterisks) and adhesion of the small intestinal loops (arrows) are persistent despite localized compression, producing a "cauliflower" appearance[24]; B: Upper gastrointestinal images reveal dilatation of the duodenum and jejunal loops, delayed bowel transit, and failure of the oral contrast to pass distally[38].

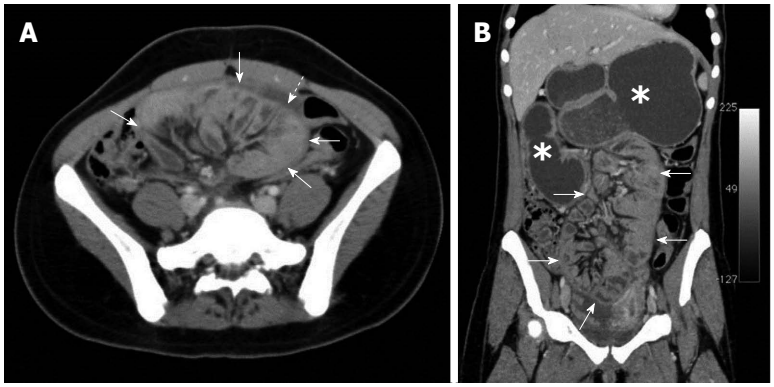

Figure 4 Contrast-enhanced abdominal computed tomography[24]. Small intestinal loops are encased in a sac of thick peritoneal membrane (continuous arrows) with a small volume of peritoneal liquid effusion (discontinuous arrow). Gastroduodenal distension is also present (asterisks). A: Axial slice; B: Multiplanar coronal reconstruction.

midline and encased by a dense capsule with a contrast-free periphery (Figure 4)[1,4,14,19,35,36]. CT may also show intestinal obstruction, ascites, localized fluid collections, peritoneal or mesenteric thickening, mural or peritoneal calcifications, and lymphadenopathy[1,60]. Multidetector CT technology has greater accuracy because it allows for multiplanar (axial, sagittal, and coronal) reconstruction. It thus provides valuable information about the severity and level of intestinal obstruction[22,40,41]. Multiplanar reformatted images provided by multidetector CT are very helpful for both exclusion of other potential causes of intestinal obstruction and planning of the surgical operation[22,29,37,41,69]. To the best of our knowledge, only one report to date has described the use of contrast-enhanced MR imaging in a patient with idiopathic SEP. Jovani *et al*[5] performed MR enterography of their patients and compared MR images with CT images after oral administration of 1.5 L of polyethylene glycol and intravenous administration of gadolinium. The authors concluded that MR-acquired images were similar to or even better than CT-acquired images in patients with SEP (Figure 5). In summary, contrast-enhanced CT (multidetector CT with multiplanar reformation) is the most helpful radiological tool for confirming the diagnosis, planning therapy, and avoiding unnecessary resection in patients with SEP.

Differential diagnosis

Most patients with symptomatic SEP present to an emergency department or general surgery clinic with recurrent acute, subacute, or chronic episodes of gastrointestinal obstruction[8,34]. Postoperative adhesions are detectable in approximately 60% to 80% of patients who present with small intestinal obstruction, while unusual conditions are diagnosed in about 6% of affected individuals[1,26,31,34,36,43,50]. Idiopathic SEP is one of the more unusual conditions that lead to intestinal obstruction[36,52,53]. Internal herniation and congenital PE are the two pathological conditions that should be primarily considered as differential diagnoses in such patients[10,16,29,43,70]. Less common conditions to be considered as differential diagnoses are voluminous invagination, intestinal malrotation, secondary peritonitis, and other causes of peritoneal adhesion[1,10,60]. Tuberculous peritonitis should be definitively excluded in patients who live in tuberculosis-prevalent regions[17,23]. Tuberculosis is so common in some regions that antituberculosis therapy is empirically administered to some patients with intestinal obstruction[23,25,27]. The medical history of the patient (*e.g.*, pulmonary or genital tuberculosis), adenosine

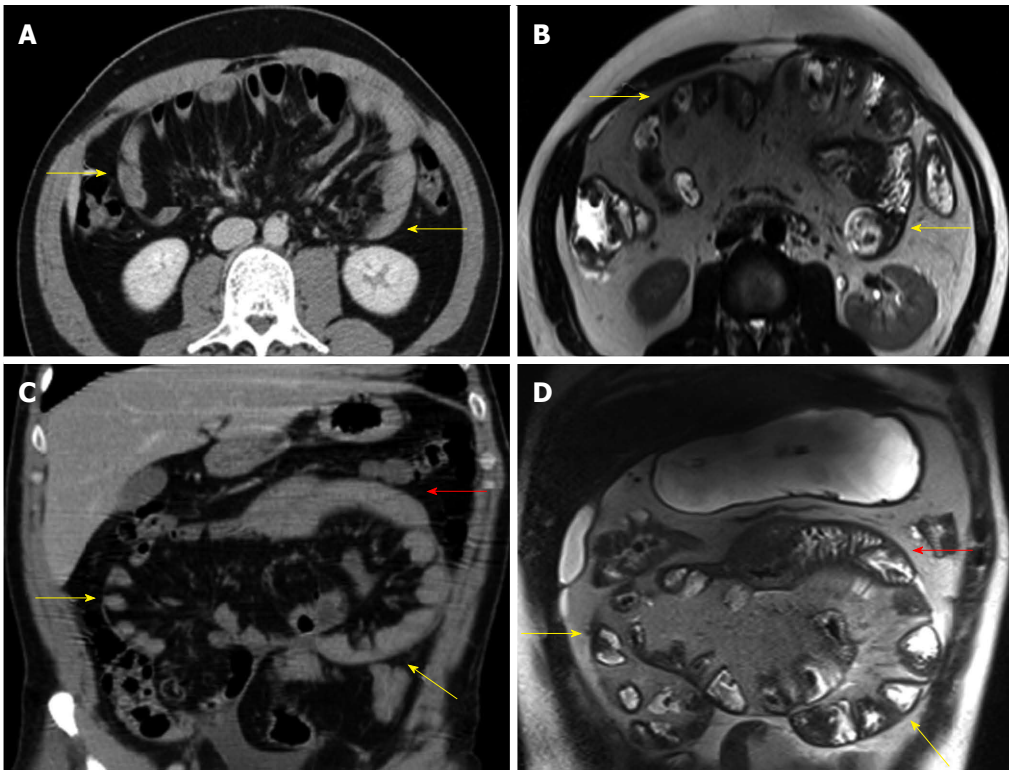

Figure 5 Comparison of diagnostic features on computed tomography and magnetic resonance images[5]. A: Computed tomography scan in the axial plane showing a subtotal conglomeration of small bowel loops coiled in a concertina-like fashion and encased by a thick membrane (yellow arrows); B: T2-weighted magnetic resonance imaging sequence in the axial plane showing bowel loops aggregated in a festoon-like shape and encased by a thick membrane (yellow arrows); C: Computed tomography scan in the coronal plane showing the conglomeration of small bowls loops (yellow arrows); a few free loops are present in the upper quadrant (red arrow); D: T2-weighted magnetic resonance imaging sequence in the coronal plane showing the same conglomerated small bowel loops (yellow arrows) and a few free bowel loops (red arrow).

deaminase level in ascitic fluid, culture of sputum and ascitic fluid, and erythrocyte sedimentation rate should be evaluated to avoid erroneous administration of clinical therapies[17,65]. Laparoscopic or open surgical biopsy of the peritoneum may be performed to rule in a diagnosis of SEP[25]. An accurate preoperative diagnosis is vital for both accurate treatment planning and prognosis prediction[1,4]. The surgeon may avoid complications more effectively when he or she knows what to expect during laparotomy[1,4,10,22,43]. However, reaching a preoperative diagnosis for many patients is a challenging task, despite the performance of an extensive preoperative radiological and clinical workup; the correct diagnosis can only be achieved by intraoperative observation and histopathological examination[36,47,57,65].

Histopathological features of SEP

The peritoneum of patients with SEP is characterized histopathologically by fibroconnective tissue proliferation, inflammatory infiltration, and dilated lymphatics. No evidence of foreign body granulomas, giant cells, or birefringent material is present. The term "sclerosing" refers to the progressive formation of sheets of dense collagenous tissue, the term "encapsulating" describes the sheath of new fibrous tissue that covers and constricts the small bowel and restricts its motility, and the term "peritonitis" implies an ongoing inflammatory process and the presence of a mononuclear inflammatory infiltrate within the new fibrosing tissue[1,41]. Although not pathognomonic, these findings are useful for the diagnosis of SEP when combined with characteristic surgical findings.

Management

There is no evidence-based consensus regarding the optimal treatment approach in patients with idiopathic SEP[8], because 97.7% of the papers on idiopathic SEP to date are case reports (1 to 6 cases). Administration of conservative treatment for as long as possible is the best approach in patients with mild abdominal symptoms. In such patients, bowel rest, nasogastric decompression, and nutritional support (enteral or parenteral) are the most appropriate treatment options[22,78]. Appetite loss, malnutrition, and weight loss are the most common symptoms in patients with idiopathic SEP[4,78]. This is because recurrent bouts of intestinal obstruction, nausea, and vomiting limit patients' oral intake, leading to weight loss and malnutrition. Li *et al*[4] showed that preoperative nutritional support is a statistically significant independent factor for preventing postoperative complications. Based on the results of their study, the authors recommended enteral nutritional support in patients who are able to eat and parenteral nutritional support in those unable to eat. Studies have indicated that enteral or parenteral

nutritional support is key to avoiding complications and malnutrition, as well as to guarantee satisfaction among patients who undergo either medical or surgical management[4,78]. Patients with symptoms resistant to conservative therapy may be treated with drug therapies comprising tamoxifen, steroids, colchicine, azathioprine and mycophenolate mofetil[1,22,38,47,79]. Corticosteroids are thought to inhibit collagen synthesis and maturation by suppressing the inflammatory process within the peritoneal membrane. They also completely eliminate the thickened membrane[78,79]. Tamoxifen is a selective estrogen receptor modulator that inhibits fibroblastic production of transforming growth factor beta, a probiotic cytokine. This drug is therefore commonly used to treat certain fibrosclerotic disorders, such as retroperitoneal fibrosis and Riedel's thyroiditis[1,26,78,79]. Many articles have described the use of tamoxifen in patients with SEP[1,26,78]. Colchicine inhibits mRNA expression of transforming growth factor beta, thereby exhibiting an anti-inflammatory action. It has a low side effect profile and cost, but a strong antifibrogenic effect[22]. Cornelis *et al*[79] reported that corticosteroids and tamoxifen are useful in preventing and/or treating SEP. However, the authors concluded that data on other agents are quite limited. Many previous studies have evaluated anti-inflammatory/antifibrogenic medical therapy in patients with SEP undergoing PD[38]. However, there are almost no data, apart from a few case reports, on the use of such medications in patients with idiopathic SEP[78,79]. Solak *et al*[22] reported the successful use of a steroid+mycophenolate mofetil in a patient with recurrent symptoms after a surgical operation for idiopathic SEP. Malik *et al*[26] similarly administered postoperative steroids. Based on the aforementioned study data, we can conclude that medical therapy may be of benefit in patients with type Ⅱ and Ⅲ cocoon syndrome in whom adequate excision + adhesiolysis cannot be achieved or in patients with recurrent postoperative symptoms.

Unlike asymptomatic/mildly symptomatic patients, those with severe signs of intestinal obstruction or who have been intraoperatively diagnosed with SEP may have several surgical options. Partial membrane excision + adhesiolysis, resection + anastomosis, resection + anastomosis + protective enterostomy, and explorative laparotomy may be used alone or in combination, depending on the patient-related factors involved[1,2,12,20,68,74]. In patients with idiopathic SEP, the most suitable procedure includes peeling the membrane off of the intestinal surface and excising the dense adhesions between the intestinal loops[4,8,75]. Membrane excision + adhesiolysis should be applied to all encased intestinal segments when there are no other contraindications for this procedure. The risk of recurrence is quite low when the membrane on the intestinal surface can be totally excised[4]. Instilling an antiadhesive substance with between the intestinal loops before closing the abdomen may prevent the development of postoperative adhesive small bowel obstruction[25,49]. Whether administration of an antifibrogenic/anti-inflammatory agent during the postoperative period is beneficial in patients in whom the membrane that encapsulates the intestinal loops cannot be completely excised is debatable. To avoid complications, such as anastomosis leakage and short bowel syndrome, in patients with idiopathic SEP, bowel resection is indicated only when necrosis has developed[1,2,4,8,63]. Resection is usually unnecessary, and, when performed without a solid indication, may increase patient morbidity and mortality[1,4,26].

The most common postoperative complications are early postoperative small bowel obstruction (EPSBO), intra-abdominal infection, enterocutaneous fistula, short bowel syndrome, and bowel perforation[4,25,34,45,56]. EPSBO usually develops within 30 d postoperatively in patients who have undergone extensive adhesiolysis and excision[56]. EPSBO is secondary to excessive manipulation of the intestinal loops, prolonged operation times, and intestinal edema[4,17,56]. It is a temporary form of intestinal obstruction that usually has no sequelae after treatment with bowel rest and total parenteral nutrition[4,17,56]. Some authors have recommended the performance of small bowel intubation through the orifice of the appendix in patients with type Ⅱ and Ⅲ cocoon syndrome to reduce the risk of developing postoperative EPBSO[4,49]. Li *et al*[4] reported that EPSBO (P = 0.0001) and adhesive intestinal obstructions (P = 0.005) were less common in SEP patients undergoing intestinal intubation. The same authors also reported that they administered nutritional support combined with somatostatin and, when necessary, low-dose steroids in patients with EPSBO[4,56]. Such a treatment approach both reduces intestinal edema and minimizes bacterial translocation caused by stasis. Spontaneous development of enterocutaneous fistulas and perforation are rare, and only one such case has been reported to date; this case was characterized by idiopathic SEP-induced spontaneous perforation[6]. Postoperative fistula and perforation, on the other hand, are secondary to iatrogenic injury or anastomosis leakage. Long-term outcomes are quite impressive in patients who have undergone appropriate membrane excision + adhesiolysis[4,8,34].

Laparoscopy is not part of the standard surgical approach in patients with SEP. A limited number of case reports have described successful laparoscopic membrane excision and adhesiolysis[37]. An advantage of laparoscopy is that it can be used for both diagnostic and therapeutic purposes in patients with an unclear diagnosis after appropriate testing (Figure 6)[17,25]. However, Hu *et al*[15] reported that when they attempted laparoscopic exploration in one patient, the trocar directly entered the bowel because of the presence of adhesions. According to both our personal experience and impressions gained from the literature, it is best to first insert the trocar into the abdomen *via* the open technique when laparoscopy is planned for treatment of intestinal obstruction or intra-abdominal space-occupying lesions[36]. This rule also applies to patients with peritoneal fibrosis secondary to SEP or other causes, as well as to patients with a history

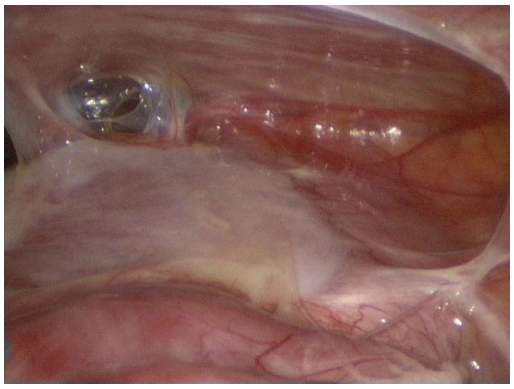

Figure 6 Laparoscopic view of the entire bowel segment encased with a fibrocollagenous membrane[37].

of abdominal surgery. Moreover, it is vital that the laparoscopic procedure is performed by an experienced operator to avoid iatrogenic bowel perforation[15,37].

In conclusion, idiopathic SEP is a clinical entity of unknown cause that is characterized by encasement of the intestines by a fibrocollagenous cocoon-like membrane. Most affected patients present to emergency departments with frequently recurring signs and symptoms of intestinal obstruction. Although recent advances in CT devices that allow for multiplanar imaging have enabled preoperative diagnosis of SEP, most cases are still incidentally diagnosed during laparotomy. Surgery remains the gold standard treatment for symptomatic idiopathic SEP. The most commonly used surgical method is membrane excision coupled with adhesiolysis. Minimally invasive management strategies help to avoid complications. Bowel rest, nasogastric decompression, and nutritional support may provide successful outcomes in asymptomatic or minimally symptomatic patients. Although various immunosuppressive, anti-inflammatory, and antifibrogenic agents reportedly provide satisfactory results in patients with secondary SEP, data on their use in patients with idiopathic SEP are limited. How those medications affect patients with idiopathic SEP remains unclear.

ACKNOWLEDGMENTS

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COMMENTS

Background

Sclerosing encapsulating peritonitis (SEP) is a chronic inflammatory process in which the small intestines are encased by a dense fibrocollagenous membrane. SEP was first described in 1907 by Owtschinnikow, who termed this condition "peritonitis chronica fibrosa incapsulata." SEP is characterized as either primary (idiopathic) or secondary, according to its underlying cause.

Innovations and breakthroughs

The primary aim of this review was to screen the literature on idiopathic SEP, also known as abdominal cocoon syndrome. To the best of our knowledge, no studies on the use of correct terminology regarding SEP, primary SEP, and secondary SEP have been performed.

Terminology

SEP is characterized by a thick, grayish-white fibrocollagenous membrane that partially or totally encases the small bowel and that can extend to involve other organs. Patients with no factors explaining the condition are considered to have primary SEP, while patients with SEP that has developed as a result of various surgical or medical conditions are considered to have secondary SEP. Based on the extent of the encasing membrane that covers the intestine, SEP is categorized into three types. Encasement of part of the intestine by a fibrocollagenous membrane is called type 1 SEP. Complete coverage of the intestine by the membrane is called type 2 SEP. Type 3 SEP refers to encasement of the whole intestine as well as other intra-abdominal organs such as the appendix, cecum, ascending colon, and ovaries.

Peer review

The study is interesting, in which authors review the literature on idiopathic SEP, also known as abdominal cocoon syndrome. The results are interesting and suggest that idiopathic SEP is a rare disorder characterized by frequently recurring bouts of intestinal obstruction.

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CASE REPORT

Rare case of intussusception in an adult with acute myeloid leukemia

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Abstract

Intussusception is rarely reported in adult patients with acute leukemia. We report a case of intussusception in a 29-year-old woman with acute myeloid leukemia (AML). She developed right lower quadrant pain, fever, and vomiting on day 16 of induction chemotherapy. Physical examination showed tenderness and guarding at the right lower quadrant of the abdomen. Abdominal computed tomography (CT) showed distension of the cecum and ascending colon, which were filled with loops of small bowel, and herniation of the ileocecal valve into the cecum. We proceeded to laparotomy and revealed ileocecal intussusception with the ileocecal valve as the leading point. The terminal ileum was thickened and invaginated into the cecum, which showed gangrenous changes. Right hemicolectomy was performed and microscopic examination of the colonic tissue showed infiltration of leukemic cells. The patient recovered after the operation and was subsequently able to continue treatment for AML. This case demonstrates that the diagnosis of intussusception is difficult because the presenting symptoms can be non-specific, but abdominal CT can be informative for preoperative diagnosis. Resection of the involved bowel is recommended when malignancy is suspected or confirmed. Intussusception should be considered in any leukemia patients presenting with acute abdomen. A high index of clinical suspicion is important for early diagnosis.

Key words: Intussusception; Acute leukemia; Abdominal pain; Colon; Malignancy

Core tip: Gastrointestinal complications are common in patients with acute leukemia, but intussusception is rarely reported in adult leukemia patients. Previous reports have mainly been in children with leukemia. We report a case of intussusception in an adult after chemotherapy for acute myeloid leukemia (AML). A 29-year-old woman with AML presented with fever, vomiting and right lower quadrant pain. Abdominal computed tomography showed features of intussusception. Resection of the involved bowel was

performed and the patient recovered from the operation. A high index of clinical suspicion is important for early diagnosis.

Law MF, Wong CK, Pang CY, Chan HN, Lai HK, Ha CY, Ng C, Yeung YM, Yip SF. Rare case of intussusception in an adult with acute myeloid leukemia. *World J Gastroenterol* 2015; 21(2): 688-693 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/688.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.688

INTRODUCTION

Gastrointestinal complications are common in patients with acute leukemia. They can be due to leukemic invasion of the bowel, an altered immune state, or the toxicity of chemotherapy[1]. Intussusception is the telescoping of one segment of the gastrointestinal tract into an adjacent one, and it is more common in children than in adults[2,3]. It is rarely reported in adult patients with acute leukemia and there is only one other case report of intussusception in an adult patient with AML, confirmed by bone marrow examination[4].

We report a case of intussusception in an adult with acute myeloid leukemia (AML). Although this complication is rare in adults, it should be considered as a differential diagnosis in patients with acute leukemia presenting with abdominal pain.

CASE REPORT

A 29-year-old woman was diagnosed with AML, confirmed by bone marrow examination, which showed AML with maturation (WHO classification) and poor-risk cytogenetics. She was given induction chemotherapy with daunorubicin 60 mg/m^2 daily for 3 d and cytarabine 100 mg/m^2 daily for 7 d. On day 16 of induction treatment, she developed right lower quadrant pain, fever, and vomiting. There was no history of prior surgery. Physical examination showed tenderness and guarding at the right lower quadrant of the abdomen, but no palpable abdominal mass. Bowel sounds were normal.

Blood tests showed that the patient's white cell count was 0.3 × 10^9/L (normal: 4.0 × 10^9-9.7 × 10^9/L), hemoglobin 7.0 g/dL (normal: 11.9-15.1 g/dL), and platelet count 15 × 10^9/L (normal: 150 × 10^9-384 × 10^9/L). Liver and renal function tests were normal, and testing for HIV antibody was negative. Blood cultures were taken and the patient was given an intravenous injection of empirical broad-spectrum antibiotic.

Abdominal computed tomography (CT) showed that the cecum and ascending colon appeared distended and filled with loops of small bowel (Figure 1). The ileocecal valve was herniated into the cecum. The wall of the ascending colon and cecum appeared thickened, and adjacent stranding was noted around the cecum, likely due to inflammation. The transverse colon appeared collapsed and the proximal small bowel was dilated with increased air-fluid level. Intussusception was suspected and emergency surgery was performed.

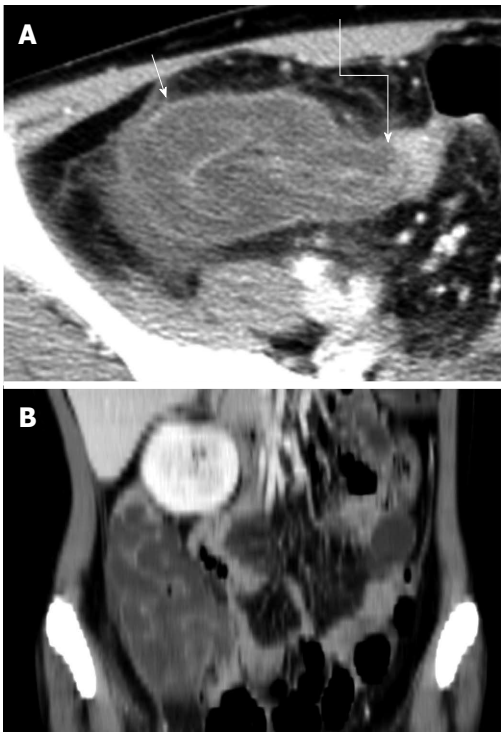

Figure 1 Contrast-enhanced computed tomography image of the lower abdomen. A: Axial image showed the terminal ileum (intussusceptum, arrow with rugged line) invaginating into the cecum (intussusceptum, arrow with straight line); B: Reformatted oblique coronal image of the iliac fossa showed thickened terminal ileum invaginating into the caecum. The wall of the cecum and ascending colon were thickened and edematous.

Laparotomy revealed ileocecal intussusception with the ileocecal valve as the leading point. The terminal ileum was thickened and invaginated into the cecum, which showed gangrenous changes. Right hemicolectomy was performed and a 5-cm long segment of ileum and a 5-cm long segment of ascending colon were examined. Macroscopic examination showed a mass 2 cm from the ileocecal junction. The mass had a whitish/brownish cut surface and was firm in consistency. The mucosal surface of the cecum and colon appeared edematous. Microscopic examination of the mass and colonic tissue showed that the submucosal and muscle layers were extensively infiltrated by leukemic cells. The cells were medium sized with irregular nuclear membranes and scanty cytoplasm (Figure 2). The cells were immunoreactive to myeloperoxidase, which is a myeloid marker.

The patient developed a wound infection after the operation. She was treated with a course of antibiotics and the wound infection improved. The patient was subsequently able to continue treatment of AML.

DISCUSSION

Intussusception is the telescoping of a proximal segment of the gastrointestinal tract within the lumen of the

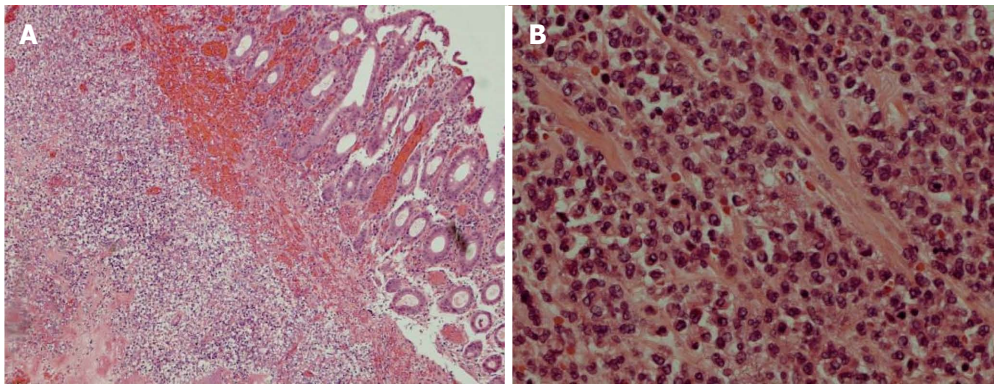

Figure 2 Histological examination of the resected colonic specimen. A: Low-power examination showed infiltration of leukemic cells into the submucosa; B: High-power examination showed that the leukemia cells were medium-sized with irregular nuclear membranes.

adjacent segment[2]. This condition is uncommon in adults and the diagnosis is usually made at laparotomy[3]. Most cases of adult intussusception involve the small or large bowel, but it sometimes occurs in the stomach or a surgically created stoma[3,5]. Intussusception may occur at sites of benign or malignant lesions, or may be idiopathic[6].

Intussusception is rarely reported in adult patients with acute leukemia, and previous reports have almost exclusively been in pediatric leukemia patients[7-15]. We identified only one other case report of intussusception in an adult patient with AML, who presented with small bowel obstruction[4]. Table 1 shows the features of previously reported cases of intussusception in acute leukemia in the literature and our present case. Adults with acute leukemia who develop intussusception present with abdominal pain or features of intestinal obstruction, whereas in pediatric patients, the clinical features are usually abdominal pain, vomiting, diarrhea and sometimes fecal blood.

A metastatic intestinal mass may act as the leading point of the intussusception[8]. Leukemia can produce a tumor mass by leukemic infiltration and hyperplasia of a polypoid nature or by intramural extravasation of blood with the formation of hematomas[7]. An intramural hematoma can be the leading point of each intussusceptum. Patients with acute leukemia usually have thrombocytopenia at presentation or after chemotherapy and are prone to hematoma formation.

Enlarged lymph nodes may also provide a leading point for intussusception in acute leukemia, because lymph node enlargement is common in patients with acute leukemia, particularly the lymphoblastic type[8]. Intussusception may also develop during induction chemotherapy from a leading point formed by leukemic filtrate, edema or necrosis[12].

The presenting symptoms of intussusception are non-specific in adult patients, but are generally chronic and consistent with partial obstruction[16]. Abdominal pain is the most common presenting symptom, followed by vomiting and bleeding from the rectum[6]. Patients may also present with an abdominal mass or intestinal obstruction[2,16].

Preoperative diagnosis of intussusception is difficult. Plain abdominal films may demonstrate features of intestinal obstruction and reveal the site of the obstruction[17]. Ultrasonography may show "pseudo-kidney" or "hay-fork" signs in the longitudinal view and "doughnut" or "target" signs in the transverse view[18]. The diagnostic accuracy of ultrasonography is dependent on the experience of the radiologist, and may be affected by obesity or the presence of massive air in the distended bowel loops.

Abdominal CT is the most useful investigation for making a preoperative diagnosis, especially in patients with non-specific abdominal pain, and it can help to assess the site and nature of the mass and the relationship to surrounding tissues. CT findings indicative of intussusception may include an apparent mass lesion, a crescent-like, eccentric, low-attenuation fatty mass or a rim of contrast material encircling the intussusceptum[19,20].

Adult intussusception requires surgical intervention[16]. However, there is still controversy regarding the extent of bowel resection, and whether reduction of the intussuscepted lesion should be attempted at operation. The potential risks of preliminary reduction of an intussuscepted bowel include perforation and seeding of tumor cells or microorganisms into the intra-abdominal cavity, venous tumor dissemination, and anastomotic complications of the edematous bowel tissue[21]. Therefore, it is recommended that resection is performed without attempting reduction when there are features of inflammation or ischemia of the bowel, or when malignancy is suspected or confirmed[21]. Our patient had ileocecal intussusception with underlying leukemia, and leukemic infiltration was suspected, so resection of the involved bowel was performed.

There have been several reports of successful surgical outcomes using the laparoscopic approach for adult intussusception[22-24]. The outcome of laparoscopic surgery is affected by the location and extent of the intussusception at diagnosis, the underlying cause, and the laparoscopic expertise of the surgeons.

In conclusion, intussusception is a rare complication

Table 1 Summary of intussusception in patients with leukemia

Number	Age/sex	Underlying leukemia	Clinical features	Imaging findings	Treatment	Clinical outcome	Ref.
1	5-yr/F	ALL	Abdominal pain, abdominal distension and constipation	X-ray showed fluid levels in bowel	Gastric suction, antibiotic therapy and supportive measures	Patient died. Autopsy showed most of the intussuscepted small bowel was gangrenous. An intramural hematoma was the leading point of the intussusceptum	Feldman *et al*[7]
2	7-yr/M	ALL	Abdominal distension	Not available	No surgical intervention	Patient died and intussusception was diagnosed at autopsy	Dudgeon *et al*[8]
3	4-yr/M	ALL	Abdominal pain, fever, vomiting and a right lower quadrant abdominal mass	Abdominal X-ray demonstrated small intestinal obstruction	At laparotomy, necrotic ileum and cecum were resected. A primary ileocolic anastomosis was performed.	Patient died with perforation of ileocolic anastomosis with peritonitis	Dudgeon *et al*[8]
4	14-yr/M	ALL	Vomiting, intermittent abdominal pain	Barium enema demonstrated an intussusception in descending colon reduced to ileocecal valve	Laparotomy showed a necrotic ileo-ileal intussusception. An ileal resection with primary anastomosis was performed.	Patient died with perforation of ileocolic anastomosis with peritonitis	Dudgeon *et al*[8]
5	11-yr/M	AML	Abdominal pain, vomiting and diarrhea	Abdominal X-ray showed air-fluid levels	Supportive treatment	Patient died of intussusception	Karakousis *et al*[9]
6	4-yr/F	ALL	Abdominal pain and vomiting	Not available	Resection of the involved bowel	Patient died of intussusception	Karakousis *et al*[9]
7	7-yr/F	ALL	Fever and colicky abdominal pain	X-ray showed dilated loops of small bowel	Surgical reduction of intussusception	Recovered from operation and continued treatment of acute leukemia	Micallef-Eynaud *et al*[10]
8	13-yr/F	ALL	Abdominal distension, abdominal pain, vomiting, symptoms of bowel obstruction	Barium enema showed small bowel intussusception	Surgical excision of the involved bowel	Recovered from operation and continued treatment of acute leukemia	Seckl *et al*[11]
9	7-mo/M	ALL	Abdominal distension, small bowel obstruction	CT scan showed small bowel obstruction	Surgical reduction of intussusception with resection of leading edge	Recovered from operation and continued chemotherapy for leukemia	Manglani *et al*[12]
10	8-mo/ F	ALL	Vomiting, blood and mucus in stool	Abdominal X-ray showed increased gas shadows in small intestine	Reduction of ileocolic intussusception	Recovered from operation and continued treatment of acute leukemia	Kumari *et al*[13]
11	3-yr/M	ALL	Abdominal pain, diarrhea, ileus	US showed thickened bowel loops with target lesion	Reduction of ileocolic intussusception	Patient died due to Escherischia coli septicemia	Gavan *et al*[14]
12	7-yr/F	ALL	Fever and colicky abdominal pain	X-ray showed a soft tissue mass in right iliac fossa. US revealed target lesion	Reduction of intussusception	Recovered from operation and continued treatment of acute leukemia	Arestis *et al*[15]
13	7-yr/F	ALL	Fever, diarrhea and colicky abdominal pain	US showed a target-shaped soft tissue mass in descending colon	Right hemicolectomy was performed	Recovered from operation and continued treatment of acute leukemia	Arestis *et al*[15]
14	25-yr/M	AML	Epigastric pain and vomiting, intestinal obstruction	Not available, but laparotomy was performed and a segment of thickened ileum which had led to ileo-ileal intussusception was found	Ileo-ileal intussusception was resected to relieve obstruction	Patient died of leukemia	Kini *et al*[4]
15	29-yr/F	AML	Right lower quadrant pain, fever, vomiting	CT scan showed that the cecum and ascending colon appeared distended and filled with loops of small bowel, and ileocecal valve was herniated into the cecum	Right hemicolectomy was performed	Recovered from operation and continued treatment of acute leukemia	Present case

16	66-yr/M	CLL	Left lower abdominal pain	US showed a large round mass in the right mid- abdomen that had alternating hypoechoic and hyperechoic rings surrounding an echogenic center (doughnut sign)	There was no surgical intervention	Patient died of leukemia	Shim *et al*[25]

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; F: Female; M: Male; US: Ultrasound; CT: Computed tomography.

in adult patients with acute leukemia. Diagnosis can be difficult because the presenting symptoms are often non-specific, but abdominal CT can be informative in making a preoperative diagnosis. Resection of the involved bowel is recommended. Intussusception should be considered in any leukemia patients presenting with acute abdomen. A high index of clinical suspicion is important for early diagnosis.

COMMENTS

Case characteristics

A 29-year-old woman with acute myeloid leukemia (AML) presented with abdominal pain, fever and vomiting, and was finally diagnosed with intussusception.

Clinical diagnosis

The clinical presentations of adult intussusception are non-specific and our patient presented with abdominal pain, fever and vomiting.

Differential diagnosis

Other causes of acute abdomen such as intra-abdominal abscess and acute appendicitis were considered and these diagnoses were excluded by imaging.

Laboratory diagnosis

Laboratory testing may not have been applicable in this case. The white cell count was not reliable because the patient received chemotherapy causing pancytopenia.

Imaging diagnosis

Contrast computed tomography showed the terminal ileum invaginating into the cecum. The wall of the cecum and ascending colon were thickened and edematous.

Pathological diagnosis

Biopsy of the resected specimen showed leukemic infiltration of the bowel.

Treatment

Most cases of adult intussusception require surgical resection because the majority of cases are secondary to a pathological condition. The patient was treated with surgical resection of intussusception.

Related reports

There is another case report of intussusception in an adult with AML who presented with epigastric pain, vomiting, and intestinal obstruction; surgery revealed ileo-ileal intussusception.

Term explanation

Intussusception is rarely reported in adult patient with acute leukemia in the literature and it is the uncommon term present in this case report.

Experiences and lessons

Intussusception should be considered in adult patients with acute leukemia presenting with abdominal pain or intestinal obstruction.

Peer review

The authors reported an interesting case of intussusception in an adult with acute myeloid leukemia occurred during induction chemotherapy. It is important to notice clinical suspicion of this entity, while this complication is rare.

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CASE REPORT

Locally advanced undifferentiated carcinoma with osteoclast-like giant cells of the pancreas

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Abstract

Undifferentiated carcinoma with osteoclast-like giant cells of the pancreas (UCOGCP) is an unusual pancreatic neoplasm that represents < 1% of all pancreatic malignancies. Moreover, the giant cells of UCOGCP morphologically resemble the benign giant cells of bone tumors. Due to the rarity of this tumor type, the histogenesis and biologic behavior of UCOGCP remain controversial. Here, we report a case of UCOGCP that exhibited an invasive growth pattern involving infiltration of the adjacent bowel loop and portal vein, as well as superior mesenteric vein thrombosis. The patient underwent a distal pancreatectomy with splenectomy and partial colectomy, followed by four cycles of gemcitabine chemotherapy. No evidence of recurrence was detected after ten years. In addition to this case, clinical information on other UCOGCP cases reported in the English literature is summarized.

Key words: Undifferentiated carcinoma with osteoclast-like giant cells; Pancreas; Locally advanced

Core tip: Undifferentiated carcinoma with osteoclast-like giant cells of the pancreas (UCOGCP) is an unusual pancreatic neoplasm and the histogenesis and biologic behavior of UCOGCP remain controversial. We report a case of locally advanced UCOGCP with infiltration of the adjacent colon and portal vein. Ten years after extended distal pancreatectomy with splenectomy and colectomy, the patient is still alive without any evidence of recurrence.

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INTRODUCTION

Extraskeletal tumors containing multinucleated osteoclast-like giant cells (OGCs), which morphologically resemble those found in giant cell tumors of the bone, are uncommon. When they do develop, they are most frequently found in the pancreas and breast. Undifferentiated carcinoma with osteoclast-like giant cells of

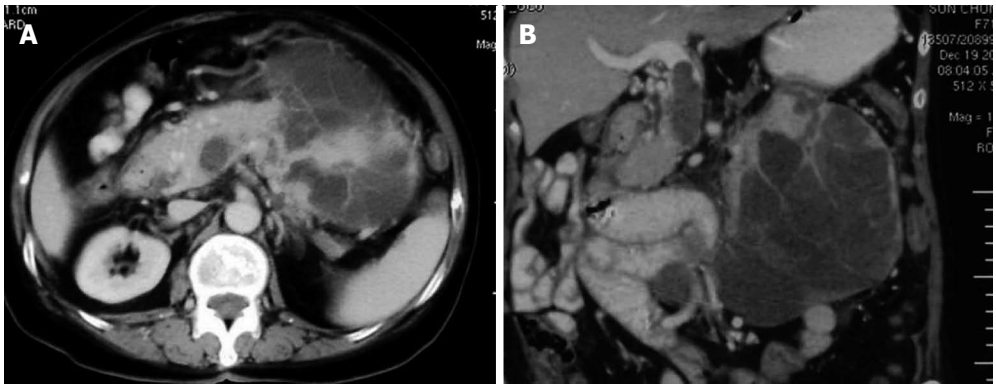

Figure 1 Computed tomography findings. A: Abdominal computed tomography showing a large heterogenous lesion extending from the body and tail of the pancreas with peripheral enhancement; B: Coronal section showing a thrombus or tumor thrombus in the portal vein and superior mesenteric vein.

the pancreas (UCOGCP) was first described by Rosai[1], and this rare tumor currently accounts for < 1% of all pancreatic malignancies[2]. UCOGCP was formerly referred to as an OGC tumor, or pleomorphic carcinoma of the pancreas with OGCs. However, UCOGCP is now classified by the World Health Organization as a rare variant of ductal pancreatic adenocarcinoma (PAC), based on the epithelial origin of its OGCs[2]. Over the last decade, the number of reports of UCOGCP has increased. However, the clinical features of UCOGCP remain obscure as many cases are already advanced when detected. For example, at the time of diagnosis, > 80% of UCOGCP tumors are > 5 cm, and 50% are > 10 cm[2]. To date, the largest UCOGCP reported was 24.5 cm[3]. In addition, UCOGCP typically includes various degrees of hemorrhage and necrosis. Herein, we report a case of UCOGCP and review the cases previously published in the English literature in order to summarize the clinicopathologic characteristics which currently describe this rare neoplasm.

CASE REPORT

A previously healthy, 71-year-old female patient was admitted due to a one month history of epigastric pain and anorexia. A 15 cm × 13 cm mass was palpated in the left upper abdomen. Laboratory examination revealed moderate anemia (Hb, 86 g/L). In addition, levels of carbohydrate antigen (CA)19-9 were 42.9 U/mL (normal, < 37.0 U/mL), CA24-2 was 22.8 U/mL (normal, < 20 U/mL), and carcinoembryonic antigen (CEA) was 2.2 ng/mL (normal, < 5.0 ng/mL). Abdominal ultrasonography and contrast-enhanced computed tomography (CECT) revealed an approximately 13 cm × 11 cm irregular cystic and solid lesion in the left upper quadrant of the abdomen. The tumor appeared to extend from the body and tail of the pancreas, and exhibited strong vascularization with peripheral enhancement that was detected by CECT. The tumor had also infiltrated the splenic hilum and adjacent bowel loop, with the splenic vein obstructed by a thrombus or tumor thrombus. Furthermore, regional lymphadenopathy, ascites, and distant metastasis were not detected in preoperative examinations (Figure 1).

During laparotomy, a large cystic and solid mass was found extending from the pancreatic body and tail, and the mass was densely adherent to the splenic hilum. The tumor had also invaded the transverse colon and partial jejunum, and extensive vascularization was observed on the surface of the tumor. The distal pancreas, spleen, and adjacent transverse colon and jejunum with their mesentery were resected *en bloc*. The splenic vein was also simultaneously opened for embolectomy. The operation time was 7 h and blood loss was 2000 mL in total.

Grossly, the resected mass was a multilocular cystic lesion measuring 17 cm along its longest dimension. On the cut surface, there was a cyst filled with necrotic and hemorrhagic content. On the inner aspect of the cyst wall, a firm nodule measuring 8 cm along its longest dimension was found (Figure 2A). Microscopically, the cyst was lined with mucinous epithelium with a few areas of high-grade dysplasia. The mural nodule consisted of variably pleomorphic cancer cells admixed with abundant and diffusely distributed multinucleated OGCs with a bland phenotype (Figure 2B). The pleomorphic cancer cells exhibited atypical, spindle-shaped, giant, and bizarre features, and these cells had densely proliferated in an inflammatory, necrotic stroma that encompassed a hemorrhagic pool. The spindle and giant cells had irregularly shaped nuclei, a thick nuclear membrane, large eosinophilic nucleoli, and abundant cytoplasm. Furthermore, these tumor cells had diffusely infiltrated into adjacent organ tissues, including the spleen, mesentery, and bowel. The OGCs lacked features of atypia, and occasionally were observed to have undergone phagocytosis of the atypical cells.

Immunohistologic examinations showed that the OGCs were strongly positive for the histiocytic marker, CD68, thereby confirming their origin from the mononuclear phagocytic system. In addition, OGCs were negative for p53, while neoplastic cells were positive for p53. No osteoid or lymph node metastasis was detected. Taken together, these results were consistent with a diagnosis of UCOGC, and according to the 6th edition of

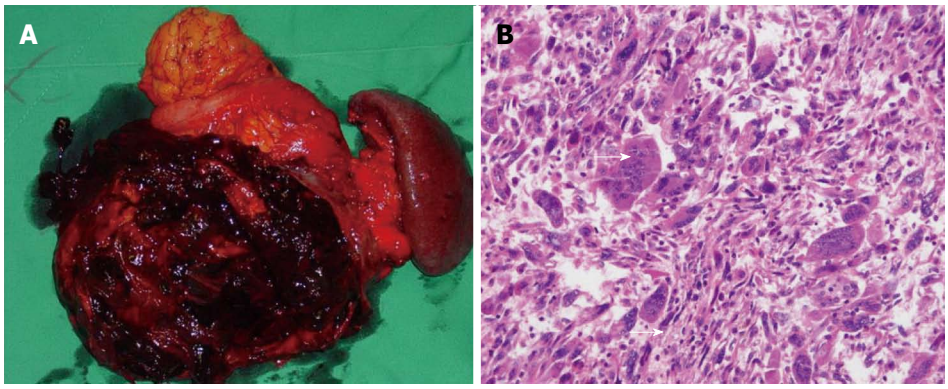

Figure 2 Gross pathology. A: Gross pathologic examination revealed a 17 cm × 12 cm mass in the pancreatic body and tail. The cut surface of the tumor included a cyst filled with necrotic and hemorrhagic content; B: Microscopically, a mixture of pleomorphic cancer cells and osteoclast-like giant cells were observed with hematoxylin and eosin staining (magnification × 20). Arrows indicate an osteoclast-like giant cell.

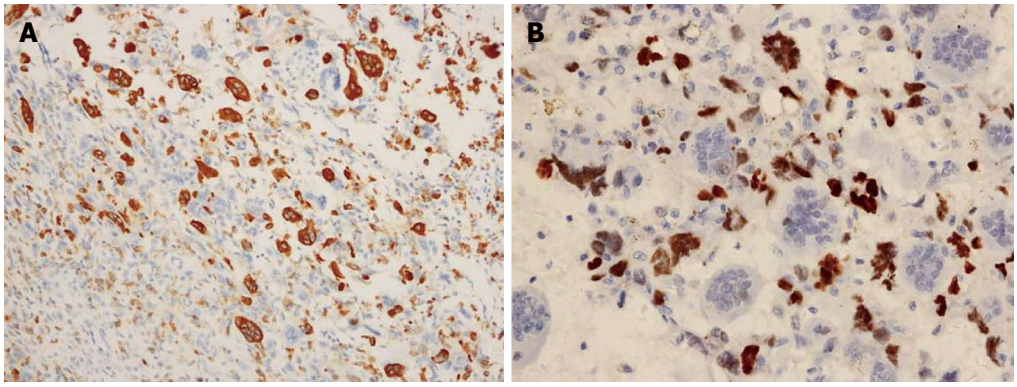

Figure 3 Immunohistochemical findings. Immunohistochemical assays detected CD68 expression in osteoclast-like giant cells (A) (magnification × 20), and p53 expression in epithelial neoplastic cells (B) (magnification × 40).

the TNM classification of the American Joint Committee on Cancer (2010), the tumor was pT3, N0, M0, stage IIB (Figure 3).

Overall, the patient remained stable throughout the operation and postoperatively, and had an uneventful recovery. The patient underwent adjuvant chemotherapy, which included four cycles of gemcitabine (1 g/m^2 on days 1, 8, and 15). CECT was performed at least annually, and cavernous transformation of the portal vein was detected four months postoperatively. For the past ten years, the patient has done well, and metastasis or any other signs of tumor recurrence have not been detected.

DISCUSSION

Histologically, UCOGCP exhibits pathological features that differ from those of common ductal PAC. In particular, UCOGCP includes highly pleomorphic, frequently spindle-shaped neoplastic cells, and large multinucleated non-neoplastic OGCs. OGCs may develop from bone marrow-derived monocytes that are recruited into the tumor by chemoattractants. Furthermore, the growth pattern and cytologic features of OGCs closely resemble those of giant cell tumors of bone. In the present report, multinucleated, benign-appearing OGCs in the neoplasm were dispersed among infiltrating pleomorphic mononuclear or multinucleated cancer cells. Immunohistologically, the OGCs were positive for the histiomonocytic marker, CD68, and were negative for the epithelial marker, p53. In addition, immunoreactivity to p53 was strong in the cytoplasm of the pleomorphic cancer cells, yet was not detected in the OGCs. These findings confirm an epithelial origin for this UCOGCP, and demonstrate that the giant cell component was not neoplastic.

Based on a review of the English medical literature, UCOGCP usually presents in the sixth or seventh decade of life, although a wide range of patient ages has been reported (*e.g.*, 32-82 years). Moreover, no gender predilection is associated with UCOGCP. The main signs and symptoms of UCOGCP include nonspecific upper abdominal pain, abdominal distension, a palpable mass, weight loss, fatigue, and anorexia. Patients with PAC also frequently present with jaundice. UCOGCP can arise from any portion of the pancreas, although it commonly develops from the body and tail of the pancreas. In contrast, ductal PAC usually involves the head of the pancreas. Similar to giant cell tumors of bone, UCOGCP is also an aggressive tumor that commonly invades adjacent organs. However, lymph node involvement and

distant metastasis are rarely observed. UCOGCP also tends to be more extensively vascularized than other cystic neoplasms of the pancreas. The sensitivity of abdominal ultrasonography, CECT, magnetic resonance imaging (MRI), and endoscopic ultrasonography (EUS) for the detection of UCOGCP are similar to those for the detection of PAC. However, while ductal PAC appears hypovascular on contrast computed tomography (CT) scans, UCOGCP appears hypervascular, which is possibly related to the rapid growth of UCOGCP, or the associated inflammatory reaction. On CT scanning, an irregular solid and cystic mass with strong enhancement are typically observed for UCOGCP. With regard to tumor markers, particularly CEA and CA19-9, elevated levels are less common, or are not distinct, in cases of UCOGCP compared with PAC. For OGCs, although they rarely express epithelial markers, they do typically stain for histomonocytic markers, especially CD68, as shown in the present report. Levels of inflammatory markers, such as white blood cell count, C-reactive protein level, and levels of interleukins, have also been found to be elevated in > 50% of patients with UCOGCP.

An accurate pretreatment diagnosis is crucial to determine the most appropriate therapy and to obtain an optimal prognosis. However, the differential diagnosis of UCOGCP from other unusual pancreatic tumors is difficult, particularly from pancreatic serous and mucinous cystic tumors, pancreatic pseudocysts, ductal pancreatic carcinomas, and neuroendocrine tumors. The presence of non-neoplastic OGCs is a histological hallmark of UCOGCP, and the diagnosis can be straightforward when examining tissue sections. Moreover, although there are limited data to support the differentiation of pancreatic lesions by CT or MRI alone, an accurate analysis of cross-sectional imaging in conjunction with clinical data may provide valuable insight into a correct diagnosis. For example, a cytologic/pathologic diagnosis is often necessary. In some cases, EUS-guided fine needle aspiration cytology (FNAC) was found to be an effective and accurate means of achieving a cytological diagnosis. For example, in a series of five patients reported by Moore *et al*[4], EUS-guided FNAC was performed, and the EUS appearance differed from that of typical ductal PAC and neuroendocrine tumors. Cytological features observed with FNAC can also distinguish primary giant cell-containing neoplasms from non-neoplastic giant cell-containing lesions of the pancreas, or giant cell-containing neoplasms that do not arise from the pancreas.

For patients with unresectable UCOGCP, the overall median survival period is 6.5 mo[5]. Therefore, the primary treatment for UCOGCP includes *en bloc* surgical resection when it is an option. However, it remains difficult to determine the best treatment modality for this neoplasm due to its rarity. An additional consideration is that more than half of giant cell tumors are locally advanced at the time of presentation, and are detected as a result of their large size or local invasion. Consequently, a partial or total pancreatectomy combined with the resection of adjacent organs is often necessary. In a previous report, a patient underwent a total pancreatectomy, and survived an additional 15 years[6]. However, an additional consideration is that the extensive vascularization that can characterize UCOGCP, as demonstrated in the present report, can lead to significant blood loss during pancreatectomy. Sporadic case reports have also demonstrated a reduction in tumor mass and prolonged survival can be achieved following treatment with 5-fluorouracil. In the present case, a chemotherapy regimen including gemcitabine was administered according to the treatment protocol of ductal PAC. Thus, it may be reasonable to consider agents such as gemcitabine for palliation. Furthermore, based on the radiosensitivity exhibited by giant cell tumors of bone, we hypothesize that radiation may also have benefits in a neo-adjuvant setting for UCOGCP. However, this remains to be evaluated in a larger cohort.

UCOGC may represent a distinct clinicopathologic entity with a more favorable prognosis than an undifferentiated carcinoma without OGCs, possibly because it is slower to metastasize and rarely metastasizes to the lymph nodes. In the literature, the outcome of UCOGCP is extremely variable, with the interval to death ranging from 4 mo to 10 years[5]. However, in a study of 35 patients, 29/35 patients did not survive, and the average survival period was 5.2 mo[7]. In contrast, for three of the patients still alive at the last follow-up, two had been disease-free for 14.6 and 7.2 years, respectively, while tumor recurrence was detected in the third patient after 14.7 years. Other studies have also reported UCOGCP patients with 10-year survival periods[7]. For the patient in the present report, she remains disease-free ten years after undergoing surgery for locally advanced UCOGCP. Correspondingly, a favorable prognosis is predicted for her long-term follow-up.

Based on the present case and limited previous data, UCOGCP is a rare malignant lesion of the pancreas that has a more favorable prognosis than ductal PAC. UCOGCP also often presents as a large cystic neoplasm accompanied by invasion into adjacent organs, and levels of tumor markers (*e.g.*, CEA and CA19-9) are usually normal or mildly elevated. For cases with advanced UCOGCP, *en bloc* resection of the pancreas and other invaded organs may be an effective treatment. It remains to be demonstrated whether the response of UCOGCP to chemotherapy or radiotherapy will be efficacious. Therefore, additional studies, preferably with larger cohorts, are needed to further improve our understanding of this rare and interesting tumor.

COMMENTS

Case characteristics

A previously healthy, 71-year-old female patient was admitted due to a one month history of epigastric pain and anorexia.

Clinical diagnosis

A 15 cm × 13 cm mass was palpated in the left upper abdomen.

Differential diagnosis

There are limited data to support the differentiation of pancreatic lesions by computed tomography (CT) or magnetic resonance imaging alone. Endoscopic ultrasonography-guided fine needle aspiration cytology was an effective and

accurate means of achieving a cytological diagnosis.

Laboratory diagnosis

Elevated levels of carcinoembryonic antigen and carbohydrate antigen 19-9 are less common, or are not distinct compared with pancreatic adenocarcinoma (PAC).

Imaging diagnosis

On CT scanning, an irregular solid and cystic mass with strong enhancement are typically observed for undifferentiated carcinoma with osteoclast-like giant cells of the pancreas (UCOGCP).

Pathological diagnosis

UCOGCP includes highly pleomorphic, frequently spindle-shaped neoplastic cells, and large multinucleated non-neoplastic osteoclast-like giant cells.

Treatment

The primary treatment for UCOGCP includes *en bloc* surgical resection when it is an option.

Term explanation

The patient described in this report remains disease-free ten years after undergoing surgery for locally advanced UCOGCP.

Experiences and lessons

For cases with advanced UCOGCP, *en bloc* resection of the pancreas and other invaded organs may be an effective treatment.

Peer review

The case presentation is interesting. For advanced cases of UCOGCP, en bloc resection of the pancreas may be an effective treatment and has a more favorable prognosis than ductal PAC. However, since this report presented as just one case, additional studies are needed to further improve our understanding of this rare and interesting tumor.

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CASE REPORT

Novel mutation in a Chinese patient with progressive familial intrahepatic cholestasis type 3

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Abstract

Genotyping is conclusive for the diagnosis of progressive familial intrahepatic cholestasis type 3 (PFIC3). Here we report a Chinese patient of PFIC3 with compound mutations in the *ABCB4* gene. Liver biopsy was performed on a 17-year-old male patient with intrahepatic cholestasis of unknown etiology. Liver histology findings are indicative of intrahepatic cholestasis with extensive fibrosis. Genotyping revealed c.175C>T (p.L59L) mutation in exon 4, c.504C>T (p.N168N) mutation in exon 6, c.711A>T (p.I237I) mutation in exon 8, c.874A>T (p.K292X) in exon 9 and a novel mutation, c.1804G>T (p.G602W) in exon 15. Based on these findings, the patient was diagnosed with PFIC3. The novel mutation p.G602W in exon 15 was predicted as probably damaging by PolyPhen-2 with a score of 0.986 (sensitivity: 0.54; specificity: 0.94) and was predicted to affect protein function with a SIFT score of 0.01.

Key words: Liver disease; Cholestasis; Progressive familial intrahepatic cholestasis; ABCB4; Gene mutation

Core tip: This study described a 17-year-old Chinese male patient with a 2 years history of intrahepatic cholestasis of unknown etiology who was later diagnosed with progressive familial intrahepatic cholestasis type 3 through clinical findings and gene analysis which revealed multiple mutations in the *ABCB4* gene. One novel mutation of the *ABCB4* gene p.G602W has also been identified. The novel mutation p.G602W in exon 15 was predicted as probably damaging by PolyPhen-2, with a score of 0.986, and was predicted to affect protein function with a SIFT score of 0.01.

Sun HZ, Shi H, Zhang SC, Shen XZ. Novel mutation in a Chinese patient with progressive familial intrahepatic cholestasis type 3. *World J Gastroenterol* 2015; 21(2): 699-703 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/699.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.699

INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) refers to a heterogeneous group of autosomal recessive

Table 1 Results of *ABCB4* gene analysis

Exon	Result	SNP No.
Exon 4	c.175 C>T (p.L59L) (heterozygous)	rs2302387
Exon 6	c.504 C>T (p.N168N) (heterozygous)	rs1202283
Exon 8	c.711 A>T (p.I237I) (heterozygous)	rs2109505
Exon 9	c.874 A>T (p.K292X) (heterozygous)	CM072813
Exon 15	c.1804 G>T (p.G602W) (heterozygous)	

SNP: Single nucleotide polymorphism.

liver disorders in childhood that disrupt bile formation and present with cholestasis of hepatocellular origin[1]. There are three types of PFIC, each caused by mutations in different hepatocellular transport system genes involved in bile formation. PFIC type 1 (PFIC1) is due to mutations in the *ATP8B1* gene; PFIC type 2 (PFIC2) is due to mutations in the *ABCB11* gene; and PFIC type 3 (PFIC3) is due to mutations in the *ABCB4* gene [also designated multidrug resistant 3 (MDR3)][2]. The non-specific clinical signs and pathological findings of this disease make the diagnosis of PFIC difficult without the use of gene analysis.

In the present report, we describe a 17-year-old Chinese male patient with a 2 years history of intrahepatic cholestasis of unknown etiology who was later diagnosed with PFIC3 through clinical findings and gene analysis which revealed multiple mutations in the *ABCB4* gene consistent with the disease.

CASE REPORT

A 17-year-old Chinese male presented with a history of pruritus and abnormal liver function for 2 years with unknown etiology by the time of admission to our hospital. No fever, weight loss, abdominal pain, rash, or joint pain was documented. The patient denies a history of hepatitis or use of any hepatotoxic drugs. He does not smoke, consume alcohol and denies any family history of hereditary or infectious disease.

On physical examination, the patient was notable for jaundice. No palmar erythema or spider angioma was noted. There was no enlargement of his superficial lymph nodes. His respiratory system and cardiac examination were normal. Abdominal examination revealed hepatosplenomegaly. Shifting dullness was absent. Examination of the musculoskeletal system was within normal limits. Lower extremity edema was negative.

Results of laboratory tests done during the time of admission were as follows: total bilirubin: 63.6 μmol/L, conjugated bilirubin: 47.9 μmol/L, total protein: 61 g/L, albumin: 34 g/L, alanine aminotransferase: 330 U/L, aspartate aminotransferase: 220 U/L, alkaline phosphatase: 495 U/L, gamma-glutamyltransferase (GGT): 388 U/L, total bile acid: 198.2 μmol/L, lactate dehydrogenase: 250 U/L. Hepatitis A, hepatitis B, hepatitis C and hepatitis E viral markers: negative. An autoantibody panel (including antinuclear antibodies, anti-smooth muscle antibodies, anti-mitochondrial antibody, anti-mitochondrial antibody M2 subtype, anti-centromere antibody, anti-histone antibodies, anti-JO-1 antibody, anti-nucleosome antibodies, anti-ribosomal P protein antibodies, anti-proliferating cell nuclear antigen (anti-PCNA) antibody, anti-polymyositis-scleroderma (anti-PM-Scl) antibody, anti-ribonucleo protein (anti-RNP) antibodies, anti-topoisomerase I (anti-SCL-70) antibodies, anti-Sm antibodies, anti-Sjogren's syndrome antigen A (anti-SS-A) antibody, anti-Sjogren's syndrome antigen B (anti-SS-B) antibody, neutrophil cytoplasmic antibody, anti-double-stranded DNA antibodies, anti-glomerular basement membrane antibodies, anti-kidney microsomal antibody type I , anti-soluble liver/liver pancreas antigen-antibody) was negative. The ceruloplasmin and tumor marker panels were within normal limits. Abdominal magnetic resonance imaging and magnetic resonance cholangiopancreatography revealed nodules in segment Ⅵ and segment Ⅷ of the liver, highly suspicious of focal nodular hyperplasia. There was also evidence of hepatosplenomegaly and portal hypertension. Liver histology obtained from a biopsy exhibited disorganized lobular structure and evidence of cholestasis, marked dilation of bile canaliculi and in some ductules containing bile plugs. Signs of chronic inflammation and fibrosis were found in the portal area. Immunohistochemical analysis with cytokeratin (CK) 7 and CK19 revealed ductular proliferation in the portal area. Reticular stain showed extensive portal fibrosis and the formation of pseudolobules. These findings are indicative of intrahepatic cholestasis with extensive fibrosis (stage 3). Genomic DNA was purified from peripheral blood. All of the 27 coding exons of ABCB4 together with at least 100 bp of the adjacent intronic sequence were amplified by polymerase chain reaction and directly sequenced. Based on the results of *ABCB4* gene analysis (Table 1), a diagnosis of PFIC3 was made.

DISCUSSION

In this study, we present a 17-year-old Chinese male patient with a novel mutation in *ABCB4*, who experienced a two years history of intrahepatic cholestasis of unknown etiology. Liver histology findings were indicative of intrahepatic cholestasis with extensive fibrosis (stage 3). We excluded other causes of cholestasis such as primary biliary cirrhosis, sclerosing cholangitis, drug induced liver injury, alpha-1 antitrypsin deficiency, cystic fibrosis, *etc*. At this point, there was a high index of suspicion of PFIC. Patients with PFIC1 and PFIC2 have normal serum GGT activity, while patients with PFIC3 have high serum GGT activity. PFIC3 patients can also be distinguished from patients with PFIC types 1-2 in that they rarely present with cholestatic jaundice in the neonatal period, but do so later on in infancy, childhood or young adulthood instead[3]. The patient's clinical features (present symptomatically at a later age), biochemical results (markedly elevated GGT) and

Figure 1 Location of the mutations relative to the respective domains in the multidrug resistant 3 protein sequence. NBD: Nucleotide binding domain.

histological findings (presence of ductular proliferation) indicated the suspected diagnosis of PFIC3 and lead to the genetic analysis of the *ABCB4* gene accordingly. Liver immunostaining of MDR3 was not considered in this patient because patients with *ABCB4* mutations exhibit variable MDR3 canalicular immunostaining[4]. Normal liver immunostaining does not preclude a gene defect, as a mutation may induce loss of function, but indicates normal synthesis and expression[3]. Furthermore, it is reported that MDR3 immunostaining on frozen liver biopsy samples is not a sensitive diagnostic tool for the detection of heterozygous *MDR3/ABCB4* gene mutations[5]. Results of the gene analysis showed that the patient is a compound heterozygote for multiple mutations reported in literature predicted to impair MDR3 function. Combined with his clinical features and laboratory findings, the diagnosis of PFIC3 was reached.

PFIC3 is caused by a genetic defect in the *ABCB4* gene located on chromosome 7[3]. MDR3 in humans are phospholipid translocators involved in biliary phosphatidylcholine excretion and are predominantly, if not exclusively, expressed in the canalicular membrane of the hepatocyte[4]. Cholestasis results from the toxicity of bile in which detergent bile salts are not inactivated by phospholipids, leading to bile canaliculi and biliary epithelium injuries. The mechanism of liver damage in PFIC3 patients is most likely related to the absence of biliary phospholipids. Injury to bile canaliculi and biliary epithelium is probably due to continuous exposure to hydrophobic bile salts, the detergent effects of which are no longer being countered by phospholipids, thereby leading to cholangitis. In addition, the stability of mixed micelles in bile is determined by a three-phase system in which the proper proportion of bile salts and phospholipids is necessary for maintaining solubility of cholesterol. The absence of phospholipids in bile would be expected to destabilize micelles and promote bile lithogenicity through crystallization of cholesterol, which might favor small bile duct obstruction[3].

According to the Human Genome Mutation Database (http://www.hgmd.org/)[6], there are over 150 disease associated mutations in the *ABCB4* gene identified, 50 of them associated with PFIC3. These include missense mutations, nonsense mutations, splice site mutations, insertions and deletions. Among them, patients with nonsense mutations and deletions of the *ABCB4* gene are associated with a more severe clinical course of the disease[7].

As reported in the sequence annotations of Uniprot database for ABCB4, accession P21439, the domains essential for organic molecule efflux are arranged along the ABCB4 protein sequence in the amino acid ranges 57-359 (1st ABC transmembrane transporter), 394-630 (1st ABC transporter), 711-999 (2nd ABC transmembrane transporter) and 1034-1279 (2nd ABC transporter). In our patient, three heterozygous silent mutations p.L59L, p.N168N and p.I237I were observed, all of which have been previously reported[8,9]. Additionally, one heterozygous nonsense mutation p.K292X and a novel heterozygous missense mutation pG602W were also found. Location of the mutations relative to their location to the respective domains in the protein sequence can be found in Figure 1.

In some patients with a PFIC3 phenotype, only one mutated allele or no mutation is identified[4]. This situation may be explained by mutations that map to the regulatory sequences of the gene. It is also possible that other unidentified genes involved in bile formation may be responsible for the PFIC1-3 phenotypes[2,3]. Modifier genes and environmental influences could also play a role in the expression of PFIC[3]. Another interesting possibility are heterozygous statuses, where a mutated protein may have a dominant negative effect on MDR3 expression or function[4].

To evaluate the functional significance of the novel mutation p.G602W, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/) were used. PolyPhen-2 uses the Naïve Bayes classifier, trained using supervised machine-learning to predict the impact of an allele replacement from its individual features and mutations are appraised qualitatively, as benign, possibly damaging, or probably damaging[10]. Using the HumVar model which distinguishes mutations with drastic effects from all the remaining human variations with a score range between 0 to 1 (values close to 0 are benign, whereas those near 1 are probably damaging), the novel mutation p.G602W was predicted as probably damaging by PolyPhen-2 with a score of 0.986 (sensitivity: 0.54; specificity: 0.94). Furthermore, multiple sequence alignment of sequences, derived from PolyPhen-2 and edited with the help of Jalview[11], showed that the novel mutation p.G602W is at a location which is physicochemically conserved in most mammals (Figure 2). Among the sequences aligned, none of them included a tryptophan at the position corresponding to glycine 602 of MDR3. All other sequences mostly harbored a glycine at the corresponding position depicting a highly conserved amino acid.

SIFT is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect. SIFT is based on the

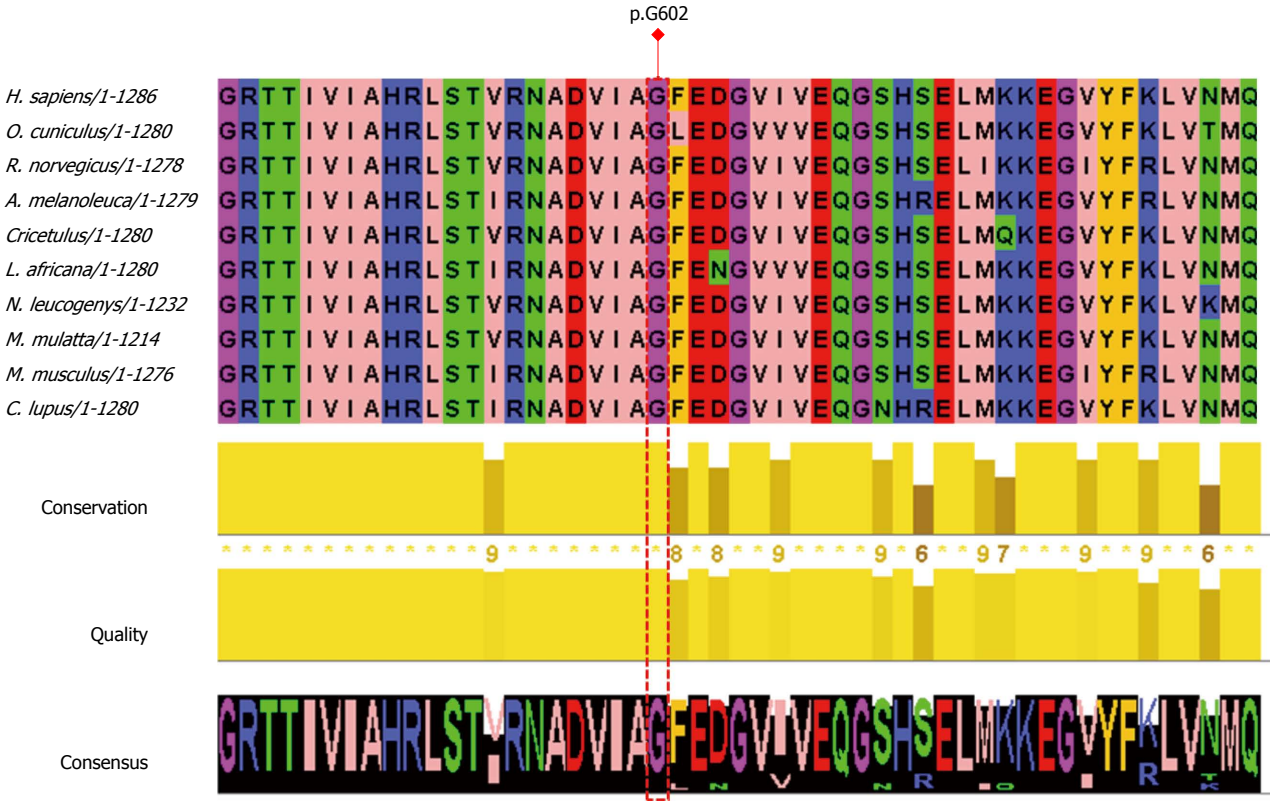

Figure 2 Multiple sequence alignment derived from PolyPhen-2 and edited by Jalview. Amino acids with similar physicochemical properties have the same color. Aliphatic/hydrophobic residues (I, V, L, A, M) are colored pink, aromatic residues (F, W, Y) orange, positive charged residues (K, R, H) blue, negative charged residues (D, E) red, hydrophilic residues (S, T, N, Q) green, conformationally special (P, G) magenta. I: Isoleucine; V: Valine; L: Leucine; A: Alanine; M: Methionine; F: Phenylalanine; W: Tryptophan; Y: Tyrosine; K: Lysine; R: Arginine; H: Histidine; D: Aspartate; E: Glutamate; S: Serine; T: Threonine; N: Asparagine; Q: Glutamine; P: Proline; G: Glycine.

premise that protein evolution is correlated with protein function. Positions important for function should be conserved in an alignment of the protein family, whereas unimportant positions should appear diverse in an alignment. SIFT predicts substitutions with scores less than 0.05 as deleterious[12]. Results from SIFT showed that the novel mutation p.G602W was predicted to affect protein function with a SIFT score of 0.01.

The patient does not have any family history and neither parent is symptomatic. With regard to clinical features, a family history for diseases associated with ABCB4 deficiency was observed in two thirds of patients harboring *ABCB4* mutations. Patients with mild genotypes, including single heterozygous mutations, have variable expression of liver disease that may be influenced by comorbidity or triggering factors and modulated by still unknown genetic modifiers[9].

In conclusion, a diagnosis of PFIC should be suspected in patients with a clinical history of cholestasis of unknown origin after exclusion of other common causes of cholestasis. Genetic analysis remains an important tool for a conclusive diagnosis of PFIC3 when combined with clinical, biochemical, radiological and histological studies. From our in silico analysis of the novel mutation p.G602W in exon 15, the highly conserved glycine 602 of human MDR3 is predicted to be essential for proper MDR3 function. This finding will be useful in future studies to further elucidate the pathogenic mechanisms of *ABCB4* mutations at the molecular level.

COMMENTS

Case characteristics

A 17-year-old Chinese male presented with a history of pruritus and jaundice for 2 years.

Clinical diagnosis

Abdominal examination revealed hepatosplenomegaly.

Differential diagnosis

The authors excluded other causes of cholestasis such as primary biliary cirrhosis, sclerosing cholangitis, drug induced liver injury, alpha-1 antitrypsin deficiency, cystic fibrosis, *etc*.

Laboratory diagnosis

Total bilirubin: 63.6 μmol/L, conjugated bilirubin: 47.9 μmol/L, total protein: 61 g/L, albumin: 34 g/L, alanine aminotransferase: 330 U/L, aspartate aminotransferase: 220 U/L, alkaline phosphatase: 495 U/L, gamma-glutamyltransferase: 388 U/L, total bile acid: 198.2 μmol/L, lactate dehydrogenase: 250 U/L. hepatitis viral markers and autoantibody panel were all negative.

Imaging diagnosis

Abdominal magnetic resonance imaging and magnetic resonance cholangiopancreatography revealed focal nodular hyperplasia in segment Ⅵ and segment Ⅷ of the liver, hepatosplenomegaly and portal hypertension.

Pathological diagnosis

Liver histology obtained from biopsy revealed ductular proliferation and portal fibrosis.

Treatment

The patient was treated with ursodeoxycholic acid.

Experiences and lessons

This case report a novel mutation in a Chinese patient with progressive familial intrahepatic cholestasis type 3 (PFIC3); the novel mutation p.G602W in exon 15 was predicted as probably damaging by PolyPhen-2 with a score of 0.986 and was predicted to affect protein function with a SIFT score of 0.01.

Peer review

The discovery and recording of a novel mutation in a Chinese patient with PFIC3 is of high clinical importance and can contribute to the recording of mutations.

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CASE REPORT

Inflammatory pseudotumor of the colon causing intussusception: A case report and literature review

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Abstract

Inflammatory pseudotumor (IPT) is a rare space-occupying lesion of unknown etiology that can mimic malignancy on clinic-radiological and pathological examination. We describe a rare case of ileocecal intussusception from clinically suspected malignancy of the right colon where the patient underwent right hemicolectomy. Histopathology of the resected specimen confirmed IPT of the colon. This patient was observed to have abnormally elevated total leukocyte count and platelets before and after surgery. In an adult with intussusception associated with an abdominal mass, the possibility of IPT of the colon should be considered. Considering the abnormally high total leukocyte and platelet counts and colonic IPT, it is necessary to prevent postoperative adverse effects due to these changes. Although IPT of the colon is usually a benign process, controversy regarding its management still exists. We consider hemicolectomy as a safe treatment approach for colonic IPT and review the existing literature.

Key words: Inflammatory pseudotumor; Intussusception; Colon

Core tip: Inflammatory pseudotumor (IPT) is a rare space-occupying lesion of unknown etiology that can mimic malignancy on clinic-radiological and pathological examination. We describe a rare case of ileocecal intussusception from clinically suspected malignancy of the right colon where the patient underwent right hemicolectomy. Histopathology of the resected specimen confirmed IPT of the colon. This patient was observed to have abnormally elevated total leukocyte count and platelets before and after surgery. We consider hemicolectomy as a safe treatment approach for colonic IPT and review the existing literature.

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INTRODUCTION

Inflammatory pseudotumor (IPT) is a reactive condition which occurs in many organs, including the lung which is the most common site of occurrence. IPT is also found in the central nervous system, major salivary glands, kidney, liver, omentum, ovary, larynx, urinary bladder, breast, pancreas, spleen, lymph nodes, skin, soft tissues, and orbit[1]. IPT is a benign tumor and represents a rare lesion with uncertain etiopathogenesis[2]. According to the location of the lesion, the clinical symptoms of IPT are diverse, and include a mass, fever, weight loss, malaise, pain and site-specific symptoms[3]. However, IPT of the colon is seldom found, and ileocecal intussusception is a rare complication of colonic IPT. We describe a rare case of ileocecal intussusception from clinically suspected malignancy of the right colon where the patient underwent right hemicolectomy. Histopathology of the resected specimen subsequently confirmed IPT of the colon and the outcome was favorable.

CASE REPORT

A 37-year-old Chinese male was referred to our Hospital with a 7-d history of intermittent abdominal pain and fever. He did not present any changes in his bowel habits, nausea or vomiting. His past medical history was unremarkable.

On admission, his axillary temperature was 37.2 ℃, heart rate was 82 beats per minute, and blood pressure was 140/90 mmHg. Physical examination showed right lower quadrant tenderness, slight rebound tenderness and localized muscle tension, no mass, shifting dullness negative, bowel sounds slightly active and reduced gurgling sounds.

His hemoglobin (HGB), total leukocyte count, platelet count and neutrophils were 134 g/L (reference range: 130-175 g/L), 13.3 × 10^9/L (reference range: 3.5-9.5 × 10^9/L), 533 × 10^9/L (reference range: 125-350 × 10^9/L) and 86.4% (reference range: 40%-75%), respectively. His random blood glucose was 7.10 mmol/L and his liver and kidney function tests were within normal limits. The patient had normal coagulation and no hepatitis B, hepatitis C, syphilis or HIV. Ultrasound examination of the abdomen showed an upper abdominal solid mass and possible intussusception (Figure 1). Computerized tomographic (CT) examination of the abdomen revealed intussusception in the right lower quadrant, possible colonic neoplasms and a right renal cyst (Figure 2). Chest X-ray showed no heart or lung abnormalities.

The patient underwent an emergency exploratory laparotomy with the presumptive diagnosis of intussusception, possible colonic neoplasms and localized peritonitis. Operative findings demonstrated a well-circumscribed, firm mass approximately 6 cm in diameter arising from the central ascending colon, intussusception and edema of the appendix. Intussusception was detected in the right abdomen and involved the terminal ileum and cecum and it could not be reset although no bowel necrosis was present. A few pieces of crisp lymph nodes (maximum diameter approximately 1.5 cm) were detected in the right mesocolon, the roots of the ileocolon and superior colic artery (Figure 3). The patient underwent right hemicolectomy with the intraoperative diagnosis of ileocecal intussusception, colonic neoplasms and localized peritonitis. Histological examination showed that the mass section diameter was about 5 cm, and the incisal surface was gray with a hard texture; microscopic

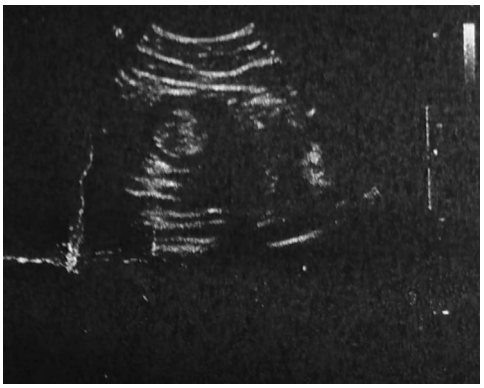

Figure 1 Ultrasound examination of the abdomen showed an upper abdominal solid mass and possible intussusception.

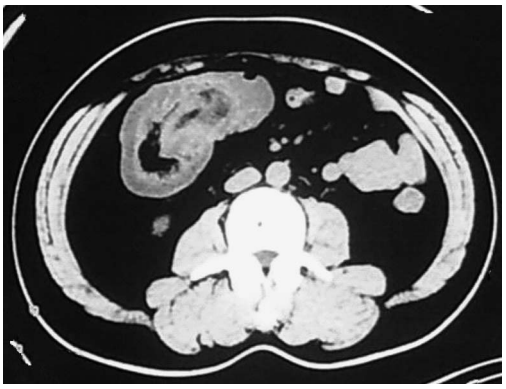

Figure 2 Abdominal computed tomography revealed an intussusception in the right lower quadrant and possible colonic neoplasms.

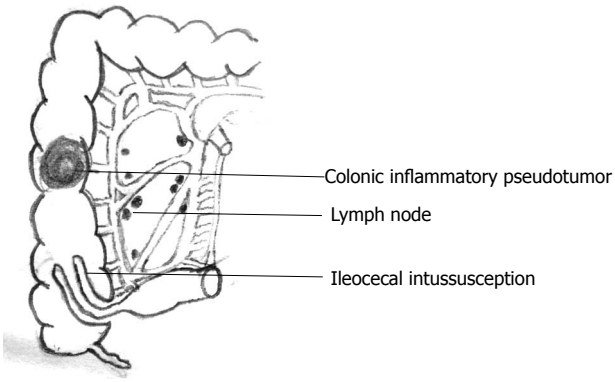

Figure 3 Schematic showing the inflammatory pseudotumor in the central ascending colon, ileocecal intussusception and enlarged lymph nodes.

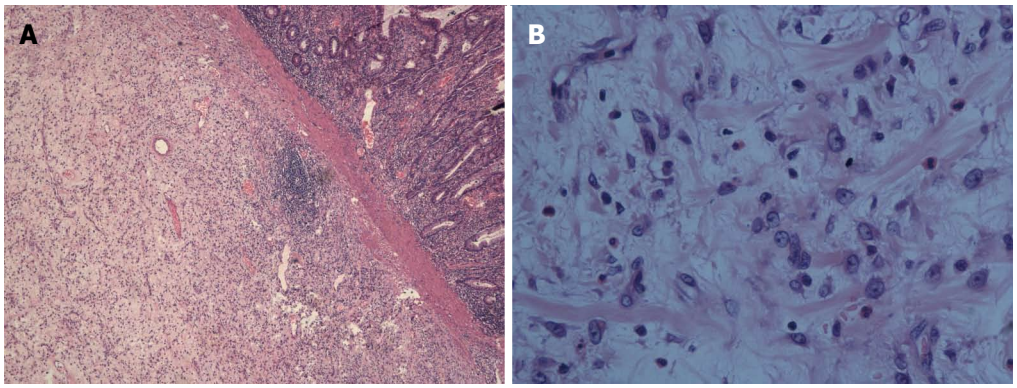

Figure 4 Microscopic examination revealed a large number of fibroblasts, myofibroblast proliferation and inflammatory changes. HE staining, A: magnification × 40; B: magnification × 400.

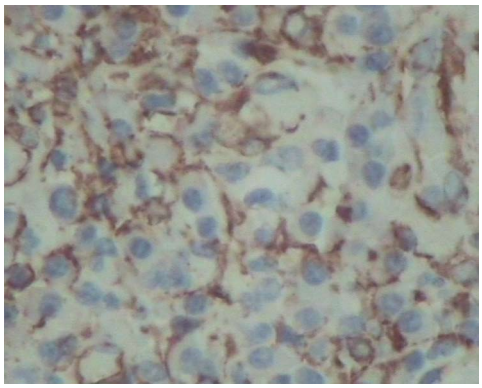

Figure 5 Immunohistochemical staining of smooth muscle actin protein was positive in the colonic mass (magnification × 400).

examination revealed a large number of fibroblasts, myofibroblast proliferation, inflammatory changes and no tumor cells (Figure 4A and B). Immunohistochemical staining for smooth muscle actin (SMA) was positive in the colonic mass (Figure 5). The histopathologic diagnosis was colonic IPT. Histopathologic examination also showed enlarged lymph nodes, follicular hyperplasia, and a significantly expanded germinal center; the histopathologic diagnosis was reactive lymphoid hyperplasia.

On postoperative day 2, HGB, total leukocyte count, platelet count, and neutrophils were 146 g/L, 24.1 × 10^9/L, 666 × 10^9/L and 90.0%, respectively. Considering the high risk of thrombosis due to the abnormally elevated platelet count, aspirin was administered to inhibit platelet aggregation. The dynamic changes in routine blood samples on postoperative day 6, 10 and 13 are shown in Figure 6A and B. During the course of leukocytosis/thrombocytosis, the patient did not have infectious signs (fever, abscess formation, *etc.*) and had undetectable C-reactive protein. Serology results showed that serum immunoglobulin G4 (IgG4) was 0.349 g/L (reference range: 0.03-2.01 g/L). Although the patient showed abnormal routine blood samples, the postoperative course was uneventful and he was discharged from hospital on postoperative day 14 and given aspirin to inhibit platelet aggregation. On postoperative day 33, his HGB, total

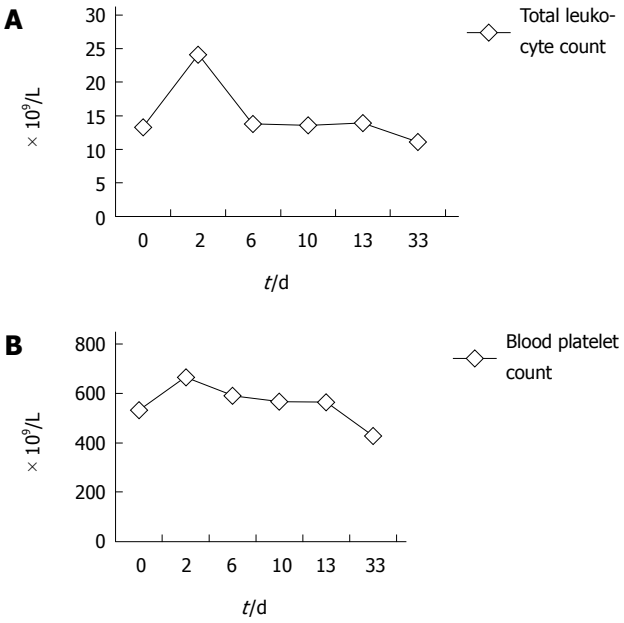

Figure 6 Dynamic changes in routine blood sample. A: Total leukocyte count (× 10^9/L); B: Blood platelet count (× 10^9/L) before and after surgery.

leukocyte count, platelet count and neutrophils were 132 g/L, 11.1 × 10^9/L, 428 × 10^9/L and 61.4%, respectively. The dynamic changes in routine blood before and after surgery are shown in Figure 6 A and B. On postoperative day 220, his HGB, total leukocyte count, platelet count and neutrophils were 154 g/L, 7.6 × 10^9/L, 312 × 10^9/L and 50.3%, respectively. The patient was free of symptoms 7 mo after surgery with normal laboratory findings.

DISCUSSION

The etiology and pathogenesis of IPT are unknown[4]. The mechanism of IPT etiology may be due to infections, intraparenchymal hemorrhage or an autoimmune etiology[5]. Microorganisms including *Bacteroides caccae, Actinomyces, Klebsiella, Escherichia coli*, Gram-positive cocci, B-hemolytic *Streptococcus*[6] and *Mycobacterium tuberculosis*[7,8] have been found in many reports of IPT. However,

Table 1 Studies on colonic inflammatory pseudotumor in PubMed database from 1994 to 2014

Position of colonic IPT	Patient number	Ref.
Indefinite	2	Aalbers *et al*[14], 1999; Velitchkov *et al*[15], 2000
Descending colon	1	Jeong *et al*[16], 2011
Transverse and descending colon	1	Fosi *et al*[17], 2014
Cecal and sigmoid flexure	2	Chetty *et al*[18], 2011
A mass in the right iliac fossa, IPT infiltrating ileocecal valve	1	Salgado-Sánchez *et al*[19], 2003
Sigmoid	3	Rosenbaum *et al*[20], 2000; De Monti *et al*[21], 1997; Wendum *et al*[22], 1994
Terminal ileum, cecum, and ascending colon	1	Cviko *et al*[23], 1999
Urinary bladder and Sigmoid colon	1	Saito *et al*[24], 1999
Colon and rectum	2	Sanders *et al*[25], 2001
Diverticular disease of the sigmoid	1	Timofeev *et al*[26], 2000
Transverse colon	2	Díaz Morant *et al*[27], 1999; Ohno *et al*[28], 1998
Cecum	1	Yoshikawa *et al*[29], 1994

IPT: Inflammatory pseudotumor.

in other reports of IPT, no causative microorganisms were found and an association between IPT and hepatopancreatobiliary autoimmune diseases, such as IgG4 sclerosing cholangitis was indicated[9]. In this case, the past medical history of the patient was unremarkable and serology results for serum IgG4 were within normal limits. Considering the abnormally elevated total leukocyte count, the etiology of colonic IPT in this patient may be associated with infection. It is regrettable that the patient, who underwent emergency surgery, did not also undergo tests for tuberculosis and other infections during the preoperative examination.

As a quasi-neoplastic lesion, the histological characteristics of IPT include a heterogeneous population of acute and chronic inflammatory cells, particularly plasma cells, macrophages and fibroblasts, accompanied by areas of fibrosis and necrosis[10]. The microscopic appearance varies from case to case. This entity has been called many different names, including plasma cell granuloma, inflammatory myofibroblastic tumor and most commonly inflammatory pseudotumor[11]. In this case, microscopic examination revealed a large number of fibroblasts, myofibroblast proliferation, inflammatory changes and no tumor cells. Immunohistochemical staining of SMA was positive in the colonic mass. The histopathologic diagnosis was colonic IPT.

Early cases of lesions classified as IPTs focused on pulmonary lesions[7,12] which were possibly more common than extrapulmonary lesions. Over the years, IPTs have also been reported at various other sites[3,13]. However, an extensive review of the literature using the PubMed database from 1994 to 2014 found only 18 cases of IPTs originating from the colon[14-29] (Table 1) and only 1 case of colonic IPT causing intussusception[16]. IPT of the colon is an extremely rare process and this unexpected lesion tends to arise from an erroneous impression of malignancy[16].

IPT is often incidentally detected on imaging studies without clinical symptoms or during diagnostic evaluation for unexplained fever, weight loss or anemia[16]. The clinical symptoms of IPT are diverse and depend on the location of the lesion. Patients with intra-abdominal tumor most commonly present with abdominal pain, a palpable mass or occasionally, with intestinal obstruction[3,13]. In this case, the patient presented with intermittent abdominal pain resulting from intussusception. In addition, due to its rarity in adults, when intussusception is diagnosed, it strongly suggests the presence of a malignant condition of either primary or metastatic origin[16]. The probability of malignancy, usually adenocarcinoma, is greater for those cases occurring in the colon[30]. In this case, the patient underwent acute exploratory laparotomy with the presumptive diagnosis of intussusception, possible colonic neoplasms and localized peritonitis. Due to cecum and ascending colon of inter-peritoneal viscera and ileum of intra-peritoneal viscera in this patient, the tumor in the central ascending colon caused bowel disorders and resulted in ileo-cecal intussusception instead of colo-colic intussusception.

IPT often cannot be differentiated clinically or radiologically from other more aggressive neoplasms, and the accurate diagnosis is based on histopathologic examination. The general appearance of abdominal IPT on ultrasound scan and tomographic scans is a well-circumscribed mass of soft tissue density producing displacement or invasion of the adjacent tissues with a homogenous echo pattern[31]. Because the radiologic findings are variable and nonspecific, it is difficult to diagnose IPT before surgery. Inconsistent radiologic images may be caused by the different degrees of inflammation and various proportions of fibrotic content within the tumor. Jeong *et al*[16] first reported 18F-FDG Positron Emission Tomography/Computerized tomography (PET/CT) in the detection of intussusception due to IPT in the colon. PET/CT application in the clinic is limited due to its high cost. In this case, CT examination of the abdomen showed intussusception in the right lower quadrant, possible colonic neoplasms and a renal cyst. The diagnosis of IPT of the colon cannot be confirmed by preoperative examination. The patient underwent acute exploratory laparotomy with the presumptive diagnosis of intussusception, possible colonic neoplasms and localized

peritonitis. Due to the firm mass arising from the central ascending colon and a few pieces of crisp lymph nodes, the patient underwent right hemicolectomy. The histopathologic diagnosis was IPT of the colon.

Patients who have IgG4-related mass lesions with dysplastic and malignant tumors endoscopically and radiographically, can undergo unnecessary invasive therapy including resection[32]. IgG4-related IPT may respond to conservative treatment with steroids[32]. However, the diagnosis of IPT is difficult due to its rarity, and the clinical history and radiographic findings lead to a high level of suspicion of a true neoplasm[5]. Generally, IPTs have a benign behavior with occasional spontaneous regression, but occasionally they have been reported to recur, metastasize, and undergo sarcomatous transformation[5]. IPT of the spleen may be diagnosed accurately by fine needle aspiration (FNA)[33]. Kawaguchi *et al*[34] also reported IPTs of the liver and spleen diagnosed by percutaneous needle biopsy. However, careful use of FNA biopsy is required in suspected intestinal IPT, as this invasive examination may cause intestinal perforation, and IPT is often misdiagnosed in pathology due to its heterogeneity and diversity. In order to avoid misdiagnosis, the patient with undiagnosed intestinal IPT should undergo exploratory laparotomy to confirm the diagnosis.

Dynamic changes in routine blood samples were noted in our patient before and after surgery. It was interesting that abnormally elevated total leukocyte count and platelets were observed before and after surgery, and these levels gradually returned to near normal with postoperative recovery of the patient. When a patient with a colon mass is observed to have abnormally elevated total leukocyte count and platelets before surgery, the diagnostic possibility of colonic IPT should be considered. Considering the abnormally high total leukocyte count and platelet count and colonic IPT, it is necessary to prevent postoperative adverse effects due to these changes. The reason for these changes is not clear. Cytokines are possibly involved in the pathogenesis of IPT[35]. Cytokines such as IL-6 and cyclin D1 probably have a paracrine action and sustain myofibroblastic growth[35]. Preoperative leukocytosis and thrombocytosis may be related to the common stimulation by inflammatory cytokines (such as IL-6) in IPT and the hematopoietic system, however, early postoperative leukocytosis and thrombocytosis may be related to surgery and anesthesia-induced trauma. The decline in late postoperative total leukocyte count and platelets is slow, late postoperative near-normal total leukocyte count and platelets may be related to removal of the IPT or the involvement of other factors. There is another possibility, in that the abnormal laboratory data is not related to IPT at all. Specific mechanisms related to the abnormally high total leukocyte count, platelet count and colonic IPT require further study.

IPT of the colon should be considered a diagnostic possibility in an adult who has an intussusception associated with an abdominal mass and has an abnormally elevated total leukocyte count and platelets before and after surgery. IPT is a rare entity that can occur in the colon in association with other inflammatory diseases. The symptoms of IPT are nonspecific, and its diagnosis is intriguing. Surgical resection is necessary and safe in many patients with IPT of the colon. Considering the abnormally high total leukocyte count and platelet changes and colonic IPT, it is necessary to prevent postoperative adverse effects due to these changes. This case report is a significant contribution to the controversy surrounding this medical problem.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

A 37-year-old Chinese male presented with a 7-d history of intermittent abdominal pain and fever.

Clinical diagnosis

Physical examination showed right lower quadrant tenderness, slight rebound tenderness and localized muscle tension, no mass, shifting dullness negative, bowel sounds slightly active and reduced gurgling sounds.

Differential diagnosis

The differential diagnosis included acute appendicitis, colonic neoplasms and right iliac fossa neoplasms.

Laboratory diagnosis

HGB, total leukocyte count, platelet count and neutrophils were 134 g/L, 13.3 × 10^9/L, 533 × 10^9/L and 86.4%, respectively: and liver and kidney function tests were within normal limits.

Imaging diagnosis

Ultrasound examination of the abdomen showed an upper abdominal solid mass and possible intussusception, and computed tomography examination of the abdomen revealed intussusception in the right lower quadrant, possible colonic neoplasms and a right renal cyst.

Pathological diagnosis

The histopathologic diagnosis was colonic inflammatory pseudotumor (IPT), which was smooth muscle actin positive.

Treatment

The patient underwent acute right hemicolectomy with the intraoperative diagnosis of ileocecal intussusception, colonic neoplasms and localized peritonitis.

Related reports

IPT is a rare space-occupying lesion of unknown etiology that can mimic malignancy on clinic-radiological and pathological examination, and its diagnosis is intriguing. Surgical resection is necessary and safe in many patients with IPT of the colon.

Term explanation

Fine needle aspiration is a method that is used for the diagnosis of solid tumors.

Experiences and lessons

This case report not only represents a rare case of ileocecal intussusception induced by colonic IPT where the patient underwent right hemicolectomy, but also revealed abnormally elevated total leukocyte count and platelets before and after surgery.

Peer review

This article presents a rare case of ileocecal intussusception induced by colonic IPT.

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