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EDITORIAL

Osteopontin as potential biomarker and therapeutic target in gastric and liver cancers

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Abstract

Gastric cancer and liver cancer are among the most common malignancies and the leading causes of death worldwide, due to late detection and high recurrence rates. Today, these cancers have a heavy socioeconomic burden, for which a full understanding of their pathophysiological features is warranted to search for promising biomarkers and therapeutic targets. Osteopontin (OPN) is overexpressed in most patients with gastric and liver cancers. Over the past decade, emerging evidence has revealed a correlation of OPN level and clinicopathological features and prognosis in gastric and liver cancers, indicating its potential as an independent prognostic indicator in such patients. Functional studies have verified the potential of OPN knockdown as a therapeutic approach *in vitro* and *in vivo*. Furthermore, OPN mediates multifaceted roles in the interaction between cancer cells and the tumor microenvironment, in which many details need further exploration. OPN signaling results in various functions, including prevention of apoptosis, modulation of angiogenesis, malfunction of tumor-associated macrophages, degradation of extracellular matrix, activation of phosphoinositide 3-kinase-Akt and nuclear factor-κB pathways, which lead to tumor formation and progression, particularly in gastric and liver cancers. This editorial aims to review recent findings on alteration in OPN expression and its clinicopathological associations with tumor progression, its potential as a therapeutic target, and putative mechanisms in gastric and liver cancers. Better understanding of the implications of OPN in tumorigenesis might facilitate development of therapeutic regimens to benefit patients with these deadly malignancies.

INTRODUCTION

Gastric and liver cancers are among the most common malignancies and leading causes of death worldwide,

which carries a heavy socioeconomic burden. Until now, surgical resection has remained the frontline treatment for patients with early stage gastric and liver cancers. Nevertheless, the majority of such patients have poor prognosis due to high rates of tumor recurrence as well as lymph node (LN) and systemic metastases. Therefore, a full understanding of gastric and liver cancers is crucial to develop useful prognostic markers and therapeutic targets. During the past decade, emerging evidence has refined the value of osteopontin (OPN) as a candidate biomarker and target for cancer therapy[1]. OPN is a secretory extracellular matrix (ECM) protein that is involved in a series of physiological and pathophysiological processes including but not limited to cell attachment, migration, invasion, proliferation, tissue remodeling, bone formation and even inflammation[1-4]. OPN is frequently overexpressed in human cancers and contributes to tumor formation and progression[5,6]. OPN belongs to the small integrin binding ligand N-linked glycoprotein family, which consists of members serving as markers of early cancer progression, due to their capabilities in modulating the activity of matrix metalloproteinases (MMPs)[7]. OPN participates in the interactions between cancer cells and tumor stroma, which plays a pivotal role in malignant cancer phenotype. A more thorough understanding of the functional role of OPN in the tumor microenvironment is warranted. There have been many reports on OPN and gastric and liver cancers, therefore, this review aims to summarize recent findings on clinical implications of OPN, its potential as a therapeutic target, and its related mechanisms in these two types of cancer. Further understanding on the role of OPN in gastric and liver cancers may facilitate development of therapeutic strategies in such patients.

OPN GENE AND PROTEIN STRUCTURE

OPN is a matrix glycoprotein secreted by a variety of cell types including osteoclasts, endothelial cells, epithelial cells, and activated immune cells such as macrophages and T cells[8]. It is also known as bone sialoprotein I, early T lymphocyte activation 1 and secreted phosphoprotein 1[9-11]. Human *OPN* gene is located on chromosome 4q21-q25, spans approximately 11 kb, and consists of seven exons encoding the OPN protein with 314 amino acid residues[12]. It contains several highly conserved structural elements, including arginine-glycine-aspartate and Ser-Val-Val-Tyr-Gly-Leu-Arg domains for integrin binding, a calcium binding site and a heparin binding domain for CD44 receptor binding[13] (Figure 1). Alternative splicing produces three OPN isoforms, OPN-a, OPN-b and OPN-c, which probably display different expression profiles and functional heterogeneity in a tumor-specific manner[14,15]. Moreover, OPN protein is subjected to a series of post-translational modifications including serine/threonine phosphorylation, glycosylation and tyrosine sulfation, resulting in molecular variants ranging from 25 to 75 kDa[16]. These modifications are cell type specific and depend on physiological and pathophysi-

ological factors, which likely affect both OPN structure and functions[17].

OPN OVEREXPRESSION AND CLINICAL VALUE IN PATIENTS WITH GASTRIC CANCER

OPN expression is significantly elevated in most gastric cancer patients at both transcriptional and translational levels[18-24]. OPN protein is overexpressed in both primary gastric cancer and metastatic lesions, mildly expressed in the epithelial cells in chronic atrophic gastritis that is a precancerous lesion for gastric cancer, and negatively in normal gastric mucosa, which indicates that OPN may play a role and serve as a potential biomarker in the formation and progression of gastric cancer[18-20]. Moreover, Wu *et al*[19] have found higher OPN plasma level in gastric cancer patients as compared with healthy individuals, suggesting that OPN plasma level may also be a biomarker for gastric cancer, and is of particular clinical interest because plasma-derived biomarkers are more convenient in clinical application than biomarkers from tissues. In gastric cancer tissues, OPN protein is diffusely located in the cytoplasm of tumor cells as well as tumor-associated macrophages (TAMs), which is in line with its implications in the interactions between cancer cells and tumor stroma.

Until now, the diagnostic and prognostic values of OPN have been implicated in gastric cancer patients. Microarray studies have identified gene signatures including OPN in gastric cancer patients[18]. OPN overexpression is significantly associated with clinicopathological parameters in gastric cancer such as low apoptotic index, high proliferative index, low grade, high stage, LN and vascular invasion, and distant metastasis[20-24]. In addition, OPN overexpression is an independent predictor of poor prognosis and tumor recurrence in patients with gastric cancer[21,22]. Dai *et al*[22] have suggested that patients with OPN-positive gastric cancer have poorer outcome than OPN-negative cases. Multivariate analysis has revealed OPN expression as an independent prognostic indicator of poor disease-free and overall survival in patients with gastric cancer, particularly for survival in cases in tumor, node, metastasis (TNM) stage II and III. The prognostic value of the marker combinations of OPN with conventional biomarkers has also been explored in gastric cancer patients. Zhang *et al*[24] have found the combination of OPN and caudal-related homeobox gene 2 (*CDX2*) as a survival predictor of advanced gastric cancer patients. OPN plasma level is commonly elevated in patients with gastric cancer, and is significantly associated with the clinicopathological features including late stage, serosal invasion, LN and vascular invasion, and liver metastasis[19]. High OPN plasma level is inversely correlated with poor prognosis in gastric cancer patients, especially in those with invasive phenotypes. Thus, elevated OPN plasma level may serve as an independent risk factor for poor survival in gastric cancer patients.
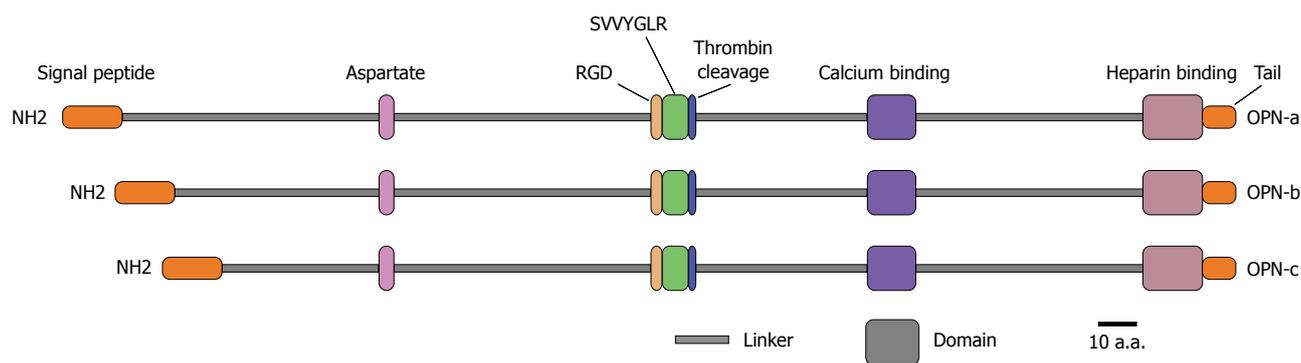
Figure 1 Structural features of osteopontin isoforms. Three isoforms of osteopontin (OPN), OPN-a, OPN-b and OPN-c, are known. All of them possess identical domains [aspartate domain, arginine-glycine-aspartate domain, Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) domain, thrombin cleavage domain, calcium binding domain and heparin binding domain] that are linked together with various linkers. These isoforms distinguish themselves by having a variable length of the linker between signal peptide and aspartate domain. RGD: Arginine-glycine-aspartate.

OPN OVEREXPRESSION AND CLINICAL VALUE IN PATIENTS WITH LIVER CANCER

OPN is positive in most hepatocellular carcinoma (HCC) patients at both transcriptional and translational levels[25-35]. Yuan *et al*[28] demonstrated OPN mRNA overexpression in 79 (51%) of 156 primary HCC patients. Kim *et al*[35] disclosed that OPN protein was expressed in 92 (32.3%) of 285 tumors. The expressions of OPN mRNA and protein display a positive correlation[29]. In HCC, OPN is secreted by both cancer cells and TAMs, and secreted by bile duct epithelium and stellate cells, but not by normal hepatocytes or Kupffer cells, in normal liver conditions[34,35]. OPN+ cancer cells are often dispersed in the periphery of cancer nodules and are adjacent to stromal cells[34,35]. In addition, OPN plasma level is also significantly elevated in HCC patients, especially in those with cirrhosis or in advanced stages[35-39]. Kim *et al*[35] determined that OPN plasma level in HCC patients was significantly higher than in patients with chronic liver diseases or healthy controls (954 ng/mL *vs* 381 ng/mL; 954 ng/mL *vs* 155 ng/mL). Zhang *et al*[36] also found that OPN plasma level of HCC patients was significantly higher than that of healthy controls (176.90 ng/mL *vs* 63.74 ng/mL). These data propose that elevated OPN plasma level can serve as a potential biomarker for HCC.

Meanwhile, several microarray studies have identified OPN-containing gene signatures of HCC patients[30-32]. Ye *et al*[31] have identified *OPN* as a leading gene in the gene signature that was relevant to tumor metastasis and patient survival. Luo *et al*[32] have found that overexpressed *OPN* gene belongs to a specific gene signature in HCC. In addition, many studies have established a significant correlation between OPN overexpression and clinicopathological features of HCC, including the severity of liver damage according to Child-Pugh class, high grade, late stage, LN/vascular/bile duct/capsular invasion, and intrahepatic or distant metastases[26-30,40-44]. Until now, OPN overexpression has been revealed as an independent prognostic factor for poor overall and disease-free survival in HCC patients[25-28,33,42-47]. In 2010, Weber

et al[42] performed a meta-analysis and found that OPN level correlated with poor overall and disease-/relapse-free survival, and as a biomarker for stage, grade, and early tumor progression in HCC. Chen *et al*[25] disclosed that OPN expression was a prognostic marker for HCC patients at TNM stage I. Furthermore, novel biomarker combinations are evaluated to predict patient outcome in HCC, since classical parameters cannot provide exact information. The biomarker combinations, OPN and α-fetoprotein (AFP), or OPN and CD44s, are revealed to have better prognostic value than the classical diagnostic biomarkers[29,44]. Huang *et al*[48] have suggested that the combination of OPN and caspase-3 can be an effective indicator for HCC patients after curative resection. However, because the published data are conflicting in many cases, further large-scale studies are necessary to confirm their clinical value[49].

Tumor recurrence is a persistent issue after surgical resection. A number of studies have suggested OPN as a useful marker for predicting early recurrence in HCC patients[25-27,33,44-46,50]. OPN polymorphisms and the combination of OPN and CD44 are potential predictors of tumor recurrence in HCC[45,46]. OPN overexpression is associated with early recurrence of hepatitis C virus (HCV)-related HCC[50]. Chen *et al*[25] found that OPN expression was correlated with early postoperative recurrence in patients at stage I. Sieghart *et al*[33] have revealed that OPN is an independent predictor of tumor recurrence and survival in HCC patients beyond Milan criteria undergoing orthotopic liver transplantation. Thus, OPN may be able to help determine the patients who need adjuvant therapy to prevent early recurrence after surgical resection.

At present, many serum biomarkers are under evaluation for the detection of HCC, but none of them has sufficient sensitivity and specificity to be considered in the guidelines. OPN plasma level increases significantly with advanced Child-Pugh class, large tumor size, high grade, and late stage[35,38]. OPN plasma level is suggested as an adverse prognostic factor for both overall survival, disease-free survival and relapse-free survival in hepatitis

Table 1 Osteopontin as a potential therapeutic target for gastric and liver cancers

Cell lines	Mouse model	Method of study	Resultant effects	Possible mechanisms
Gastric cancer				
SGC7901	Nude mice	siRNA knockdown	Reduced angiogenesis *in vitro* and *in vivo*	Decreasing microvessel density
BGC-823	Nude mice	Transient/stable siRNA knockdown	Inhibited cell growth, anchorage-independent growth, migration and invasion *in vitro*, and suppressed tumor growth and prolonged survival *in vivo*	Inhibition of MMP-2 and uPA expression, NF-κB DNA binding activity, and Akt phosphorylation
SGC7901	Nude mice implanted with SGC-OPN-cells	Lentivirus-mediated stable depletion	Suppressed metastases and prolonged survival time *in vivo*	Reducing expression of VEGF
HCC				
MHCC97-L, MHCC97-H, HCC-LM3		siRNA knockdown	Decreased cell invasion and cell cloning number *in vitro*	
HuH1/4/7, MHCC97, SMMC7721, SK-Hep-1, Hep3B, CCL13, HCCLM3	Nude mice of lung metastasis	OPN-neutralizing antibody	Blocked invasion of SK-Hep-1 and Hep3B cells *in vitro*, inhibited pulmonary metastasis of HCC-LM3 cells *in vivo*	
HCC-LM6	Nude mice implanted with HCC-LM6	Antisense knockdown	Suppressed migration and invasion *in vitro*, decreased lung metastases *in vivo*	Inhibiting MMP-2 and uPA expression
HCC-LM3	Nude mice implanted with Lenti OPNi-transfected HCC-LM3 cells	Stable depletion using lentiviral vectors encoding miRNA against OPN	Inhibited both *in vitro* proliferation, invasion and *in vivo* tumor growth and lung metastasis	Inhibiting MAPK and NF-κB pathways, and MMP-2 and suppressing uPA expression
HCC-LM3 HepG2	Nude mice	*shRNA* gene silencing	Inhibited HCC cell growth, adhesion and invasion *in vitro*, and suppressed tumorigenicity and lung metastasis *in vivo*, enhanced sensitivity of HCC cells to chemotherapeutic drugs	Suppressing αv, β1, β3 integrin expression, blocking NF-κB activation, inhibiting apoptosis

HCC: Hepatocellular carcinoma; OPN: Osteopontin; siRNA: Small interfering RNA; MAPK: Mitogen-activated protein kinase; NF: Nuclear factor; MMP: Matrix metalloproteinase; VEGF: Vascular endothelial growth factor; uPA: Urokinase-type plasminogen activator; shRNA: Short hairpin RNA; Akt: Protein kinase B; miRNA: microRNA .

B virus (HBV)- or HCV-related HCC patients[36,38,51]. In addition, OPN plasma level may be a potential diagnostic biomarker for HCC in the surveillance of patients with HBV or HCV infection. Sun *et al*[51] have suggested that preoperative plasma level of OPN and AFP can be used as a prognostic marker for early stage HCC. A recent study conducted by Shang *et al*[52] has also identified serum OPN as a novel marker for early HCC diagnosis because OPN was found clearly elevated 1 year before diagnosis in a pilot prospective study including 22 patients. In another two studies, a greater area under curve value of OPN than AFP was observed, suggesting superior diagnostic accuracy of OPN for HCC[38]. HCC patients whose pretreatment OPN serum level is low and declines following transarterial chemoembolization exhibit better tumor response and longer survival[37]. These data suggest that OPN plasma level can be used, either independently or coupled with AFP, for predicting clinical outcome in HCC patients.

OPN AS A POTENTIAL THERAPEUTIC TARGET FOR GASTRIC AND LIVER CANCERS

OPN as a therapeutic target has been explored in various tumors including cancers of breast, lung, head and neck, stomach, colon and liver. Promising results have been achieved in a series of studies[53-57]. The strategies often utilize OPN antibody to block its binding to receptors so as to inhibit the downstream signal transduction related to tumor growth and invasion, and deliver the small interfering RNA (siRNA) targeting OPN to tumor cells to decrease directly the expression of OPN to abrogate the effects triggered by elevated OPN.

At present, OPN-knockdown-induced tumor suppression in gastric cancer has been shown through RNA interference (RNAi)[58-60]. *In vitro* and *in vivo* studies have demonstrated OPN-RNAi-induced inhibition of tumor growth, migration and invasion in gastric cancer[58,59]. Moreover, Wang *et al*[60] silenced OPN expression in gastric cancer cell line SGC7901 using lentiviral-OPN siRNA technology, and found reduced detectable tumors, fewer metastases, and longer survival time in mice implanted with OPN-SGC7901 cells. These data suggest that targeting OPN and its related signaling network is likely to provide an effective therapeutic approach for gastric cancer (Table 1).

In recent years, efforts have also been made to inhibit HCC progression and metastasis by interfering OPN[27,31,61-63]. OPN knockdown significantly suppresses migration and invasion of HCC cells *in vitro* and decreases lung metastases *in vivo*, which is associated with decreased angiogenesis in HCC cells[61,62]. Besides, OPN-specific antibody can effectively block HCC cell invasion *in vitro* and inhibit lung metastasis of HCC cells *in vivo*[31]. In addition, Zhao *et al*[63] have demonstrated that short hairpin RNA-mediated OPN depletion enhances sensitivity of HCC cells
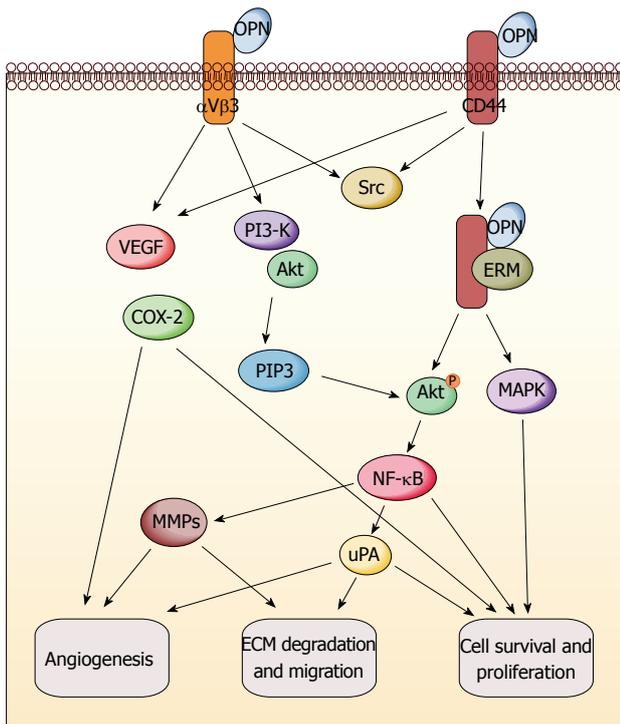
Figure 2 Molecular mechanisms of osteopontin in gastrointestinal cancers. Osteopontin (OPN) signaling leads to gastrointestinal cancer growth and metastasis through activation of various pathways, including cell survival and proliferation, angiogenesis, and extracellular matrix (ECM) degradation. VEGF: Vascular endothelial growth factor; PI3-K: Phosphoinositide 3-kinase; COX-2: Cyclooxygenase-2; MAPK: Mitogen-activated protein kinase; NF: Nuclear factor; uPA: Urokinase-type plasminogen activator; MMP: Matrix metalloproteinase; ERM: Ezrin/radixin/moesin; Akt: Protein kinase B.

to chemotherapeutic drugs through blockade of nuclear factor (NF)-κB activation. Thus, targeting OPN and its related signaling network is likely to help develop novel therapeutic regimens for HCC (Table 1).

MOLECULAR MECHANISMS OF OPN IN GASTRIC AND LIVER CANCERS

The multifunction of OPN has been revealed in promoting tumor formation and progression (Figure 2). It exerts these functions through direct binding to integrin and/or CD44. The subsequent activation of various pathways leads to increased malignant phenotype[64,65]. Various signaling transduction pathways triggered by OPN molecule have been reported in different cancer models such as breast cancer, melanoma, lung cancer, myeloma, prostate cancer and gastrointestinal cancers. The results indicated that OPN exerts the tumor-related functions through a complicated signaling network[65]. Here, we only summarize the reported signaling pathways of OPN relevant to gastric and liver cancers; some of which are commonly overlapped with other cancers, but some are specific in these two types of cancers. It has been suggested that phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt) pathway and hypoxia-inducible factor-1 are involved in the tumor-promoting function of OPN,

which induces pro-survival and anti-apoptosis signaling in gastric and liver cancers after the survival pathway is activated[63,66]. Mitogen-activated protein kinase pathway (MEK/ERK1/2) can also be triggered by OPN protein in liver cancer to promote tumor growth and metastasis, while the effect can be reversed through OPN knockdown[62]. The NF-κB pathway is crucial to keep cell survival through initiating the gene expression of antiapoptotic proteins, and is often induced by chemotherapeutic drugs and contributes to resistance to chemotherapy[59,62]. Relevant tumor-promoting functions of OPN are found to be highly associated with NF-κB pathway activation in gastric and liver cancers[59,62,63]. The MMP family is responsible for ECM degradation and remodeling, which play an important role in tumor invasion and metastasis. OPN-induced metastasis of gastrointestinal cancers is also involved in several MMP members such as MMP-2, MMP-9, MMP-7 and other famous invasion-related proteins such as vascular endothelial growth factor (VEGF) and urokinase-type plasminogen activator (uPA)[54,59,62,67,68]. Recently, Lee *et al*[66] illustrated that OPN can enhance the survival of gastric cancer through the interaction with CD44 variant isoforms. The underlying mechanism involves Src kinase signaling upon OPN binding to CD44, followed by "inside-out" integrin activation. In addition, there may be a positive correlation between OPN and cyclooxygenase-2 (COX-2). OPN, VEGF and COX-2 could synergistically induce angiogenesis and metastasis in gastric cancer[69]. On the other hand, the antitumor activity of COX-2 inhibitors in intestinal cancer is probably mediated through downregulation of OPN, which results from blockade of nuclear receptor subfamily 4, group A, member 2 (NR4A2) and Wnt/β-catenin signaling, two important components of the OPN regulatory network[70].

Several mechanisms regulating *OPN* gene expression have been revealed, but many details remain to be elucidated. OPN is a transcriptional target of aberrant Wnt/β-catenin signaling[70-72], and is also regulated by other molecules including specificity protein 1, v-ets erythroblastosis virus E26 oncogene homolog 1, runt-related transcription factor 2, v-myb myeloblastosis viral oncogene homolog, CDX2, deleted in liver cancer 1, late SV40 factor (LSF), epidermal growth factor (EGF), NR4A2 and NO[24,73-79]. Interestingly, the activation of several downstream targets of OPN, such as Akt, LSF, NO, EGF and thrombin, can enhance OPN expression in turn, suggesting a positive feedback regulation of *OPN* gene expression[68,73,78-82]. Moreover, the modulation of OPN mRNA stability also influences OPN expression in HCC[83,84]. In addition, miRNA-181a decreased OPN expression in HCC cell lines, suggesting that miRNA is involved in the regulation of *OPN* gene expression[85]. Furthermore, the expression of OPN is also affected by COX-2 and 30-kDa Tat-interacting protein[70,86,87].

In short, OPN signaling could result in the activation of anti-apoptosis and pro-survival pathways *via* PI3-K-Akt and NF-κB signaling molecules, angiogenesis modulation *via* VEGF induction, ECM degradation *via* MMPs

and uPA secretion, leading to tumor growth and metastasis in gastric and liver cancers.

CONCLUSION

OPN overexpression occurs frequently in patients with gastric cancer and liver cancer. Previous studies have revealed its clinicopathological correlation with tumor formation and progression in these two types of cancer, indicating its potential as an independent indicator for predicting outcome in such patients. Functional studies have shown the potential of OPN as a therapeutic target in gastric and liver cancers both *in vitro* and *in vivo*. OPN mediates multifaceted roles in the interaction between cancer cells and tumor microenvironment, in which many details need to be further explored. The various mechanisms of OPN signaling in gastric and liver cancers including evasion of apoptosis, modulation of angiogenesis, ECM degradation, activation of PI3-K-Akt and NF-κB pathways, might induce the development and progression of gastric and liver cancers. However, no clinical trial targeting OPN is in progress for tumor treatment, although the importance of OPN has been widely investigated and demonstrated in various cancers, and many patents including antibodies or peptides against OPN have been filed to treat different tumors. OPN is an important cytokine to mediate normal physiological functions. Blocking OPN possibly results in severe adverse effects due to interference with normal OPN roles. Therefore, further understanding of the implications and roles of OPN in various tumors including gastric and liver cancers could help develop better therapeutic strategies for such patients. On the other hand, OPN as a secreted plasma protein seems to have a greater potential to be utilized as a diagnostic or prognostic marker for in relevant cancers in combination with other biomarkers or alone.

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S100A4 in esophageal cancer: Is this the one to blame?

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Abstract

Metastasis is the main reason for cancer-related death. S100A4 is one of the key molecules involved in this event. Several studies have shown that overexpression of S100A4 in non-metastatic cancer cells can make them become metastatic, and knockdown of S100A4 in metastatic cancer cells can curtail their invasive nature. A study by Chen *et al*[2] published in the *World J Gastroenterol* 18(9): 915-922, 2012 is a typical example. This study showed *in vitro* and *in vivo* evidence that S100A4 expression level determines the invasiveness of esophageal squamous carcinoma. Considering the fact that more than half of the cancer-related deaths are caused by malignancies derived from the digestive system and esophageal cancer is the 4th top contributor to this fraction, this study warrants more attention.

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Key words: Esophageal cancer; S100A4; Metastasis

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INVITED COMMENTARY ON HOT ARTICLES

Cancer is the second leading cause of death in the world (18%), after heart disease (21%). Among about 18 million new cases of cancers diagnosed each year, about one third is skin cancer. However, 95% of skin cancer is either basal cell carcinoma or squamous cell carcinoma, which has a mortality of less than 0.5%. The majority of cancer-related deaths are actually caused by malignancies derived from the digestive system, including esophagus, stomach, small intestine, colon, rectum, anus, liver, gallbladder and pancreas[1]. The main feature that makes these cancers deadly is metastasis, a process that cancer cells break off from their original location and invade other parts of the organ. The majority of skin cancers do not have this capacity; therefore, they can be easily treated before becoming life threatening. Esophageal cancer, on the other hand, is highly metastatic. Therefore, understanding the molecular mechanisms behind its metastasis is of great values for developing better treatment strategies. A study by Chen *et al*[2] published in the *World J Gastroenterol* 18(9): 915-922, 2012 examined the role of S100A4, one of the well-known cancer metastatic markers, in esophageal squamous cell carcinoma (ESCC) *in vitro* and *in vivo*, in animal models as well as in clinical human specimens, and clearly demonstrated a reliance of the invasiveness of esophageal cancer on this small calcium-binding protein[2].

A little biography of S100A4: Short but hot

S100A4 was discovered in the mid 1980s by several laboratories independently. One of these laboratories be-

longed to Daniel Nathans, MD (10/30/1928-11/16/1999) (Figure 1), the Nobel Prize winner in Physiology/Medicine 1978 for his landmark discovery of restriction enzymes. In 1983, one of his post-doctoral fellows, Daniel I Linzer, PhD, was constructing a cDNA library from serum-stimulated mouse 3T3 fibroblasts and found that a clone named 18A2 was highly up-regulated by serum exposure[3]. There seemed to be many laboratories in the late 70s and early 80s of the 20th century which were interested in the effect of serum on gene expression. That was also how and when serum response factor (SRF) was discovered[4,5]. In the following year, Linzer took a job at Northwest University in Illinois (now he is the Provost of this school) and continued his study on 18A2. He determined that 18A2 coded for a calcium binding protein of 101 amino acids[6], much similar to the members of S100 family, a group of small peptides that are known to be 100% soluble in saturated ammonia sulfate. He also compared the sequence of 18A2 with 2A9, a human clone that was published a year earlier[7], and found a 57% nucleotide and 62% amino acid homology between them. It might be due to the difference of species origin, Linzer was pretty sure that these two sequences represented different genes. Around that time and shortly thereafter, several other laboratories also published similar sequences and each of which was given a different name, including p9Ka from rat mammary cells[8], 42A from rat neuronal cells[9], pEL98 from mouse fibroblasts[10], CAPL from Aplysia neurons[11], mts1 from metastatic tumor cells[12], and FSP1 from mouse fibroblasts[13]. Despite the individuality of each of these studies, there were some common features shared among their discoveries: (1) serum inducibility; (2) around 100 amino acids; and (3) similarity to S100 calcium binding proteins. Although all of these sequences eventually turned out to be for a single molecule - S100A4, each of these studies made unique contributions to our knowledge today about S100A4. The last two studies warrant an extra attention, because one established the connection between S100A4 and cancer metastasis and the other associated it to fibroblast phenotype. Now we know that S100A4 is a prognostic marker for metastatic cancers as well as a marker for epithelial-mesenchymal transition. However, both of these studies went a little bit too far by calling this molecule metastatic-specific and fibroblast-specific, respectively. Now we know that is not entirely true, a lot of other cells (e.g., epithelial cells, endothelial cells, lymphocytes, smooth muscle cells, *etc*.) also express S100A4, just as our study reported[14].

Functions of S100A4: Motivation to move

Up to date, S100 family includes 25 members with common characteristics such as low molecular weight, two calcium binding sites of the helix-loop-helix ("EF-hand type") conformation, and complete solubility in ammonium sulfate at pH 7. They have been implicated in regulation of protein phosphorylation, transcription factor activation, calcium homeostasis, cytoskeleton reorganization, cell migration, cell growth and death[15].

Figure 1 Daniel Nathans, MD, Nobel Laureate (10/30/1928-11/16/1999), Department of Molecular Biology and Genetics, the Johns Hopkins University School of Medicine Baltimore, Baltimore, MD 21205, United States.

S100A4 is naturally expressed in various cell types including both cancer and normal cells, and its elevation is usually associated with cell motility. It appears that wherever cell migration is required, such as wound healing[16], angiogenesis[17] and cancer metastasis[18], S100A4 is activated. Like other members of S100 family, S100A4 works like a calcium sensor. Upon calcium binding, S100A4 goes through a series of conformational changes, which allow the molecule to interact with its targets, such as nonmuscle myosin heavy chain (MHC ⅡA) and liprin β1, to facilitate cell migration[19,20]. For this reason, in motile cells, S100A4 is often found in complex with these cytoskeletal components at the migrating front where a high level of calcium is accumulated. It is interesting to know that S100A4 knock-out mice do not display developmental abnormalities in the postnatal period, but 10% of them develop tumors at age of 10-14 months, possibly due to destabilization of the tumor suppressor p53[21], as S100A4 has been shown capable to bind to the C-terminal of p53 and repress its transcriptional activity[22,23].

Yet, the story of S100A4 is not as straightforward as it might have been anticipated. In addition to being a cytoskeletal regulator in the cytoplasm, S100A4 has also been localized to the nucleus and extracellular matrix. How it gets there and what it does in these locations remain unclear. Nevertheless, its association with transcription factors like p53 might explain some of its roles in the nucleus. It has been postulated that S100A4 binding to the tetramerization domain of p53 favors p53 oligomerization and thereby facilitates p53 nuclear translocation[23]. On the other hand, extracellular S100A4 has been demonstrated to stimulate MMP-13 expression in chondrocytes in a receptor for advanced glycation end products (RAGE)-dependent manner[24], while its inductivity on neuron growth was found to be RAGE irrelevant[25]. More complicatedly, S100A4 has been found in association with cell death in a conflict way, it inhibits apoptosis in pancreatic cancer[26] but promotes it in osteosarcoma cells[27].

S100A4 in cancers: A facilitator, not a generator

Elevation of S100A4 has been found in almost every metastatic cancer known, including breast[28], ovarian[29], prostate[30], urinary bladder[31], lung[32], esophageal[33], gastric[34], colon[35], pancreatic[36], liver[37], gallbladder[38] and

thyroid carcinomas[39]. More direct evidence for the essential role of S100A4 in cancer metastasis perhaps comes from *in vitro* studies and animal models, which have shown that overexpression of S100A4 in non-metastatic tumor cells confers a metastatic phenotype, just as demonstrated in the study by Chen *et al*[2] as well as several others[40,41]; whereas, knockdown of S100A4 in metastatic tumor cells curtails their invasive capability[2,42,43].

It should be pointed out though that S100A4 is not an oncogene product. As shown by transgenic studies[17,44], mice carrying extra copies of *S100A4* gene develop normally as wild-type and have no increased risk of cancer. However, when these mice mated with cancer mice, their offspring showed increased number of tumors distant from their primary location[45]. Therefore, S100A4 is not a cancer generator but a metastatic facilitator.

S100A4 has been studied extensively in other cancers, especially in breast cancer. In esophageal cancer, there are about a dozen of publications so far, mostly focusing on squamous cell carcinoma. The earliest study that can be found was done by a Japanese group[33], showing an elevated expression of S100A4 protein in surgically resected ESCC, and a possible association with esophageal cancer progression. However, a later study reported an opposite result, showing that 11 out of 16 S100 family members examined, including S100A4, were down-regulated at transcriptional level in tumor tissues compared with adjacent normal tissues[46]. In 2010, a Chinese research team used RNA interference technology to knock down S100A4 in metastatic esophageal tumor cells and grafted them in nude mice[47]. They noticed that tumor growth was significantly inhibited by S100A4 deficiency, and E-cadherin expression was reciprocal to the level of S100A4. Unfortunately, the study had little impact because it was published in a local journal in Chinese. However, the idea of xenografting has recently advanced to a new cancer treatment strategy - the "avatar" mice. Principally, it is to take tumor tissue from a patient and graft it in nude mice to create a personalized colony of mice carrying exact that patient's cancer, and then test every potential treatment combinations in mice before selecting the best one to treat that patient. Manuel Hidalgo, the Director of the Spanish National Cancer Research Center in Madrid, has been practicing this approach for pancreatic cancer patients over years and showed a clear advantage in drug responses[48,49], and now more and more researchers believe that this idea holds a great promise in cancer treatment in the future.

In the study by Chen *et al*[2], the research team cleverly used two ESCC cell lines, EC109 (highly invasive) and TE13 (non-invasive), and successfully made these cells switch characters by down-regulation of S100A4 in EC109 and up-regulation of S100A4 in TE13. They provided *in vitro* and *in vivo* evidence that the level of S100A4 determines the metastatic status of the cancer.

There are two main subtypes of esophageal cancer: ESCC and esophageal adenocarcinoma (EAC). Although nearly 95% of esophageal cancer is ESCC, EAC has been rising by 6-fold annually in Americans and now its in-

crease rate exceeds the rate for any other type of cancers. Overexpression of S100A4 was also reported in EAC and its correlation with lymph node metastasis was found significant[50].

Although the exact molecular mechanisms how S100A4 promotes cancer metastasis still need to be further examined, based on various studies, one possible explanation could be that S100A4 binding to liprin β1 inhibits its phosphorylation[19], and thereby prevents its interaction with liprin α1. As a result, liprin α1 fails to recruit leukocyte common antigen-related (LAR) protein[51], a phosphatase, to focal adhesions. Without LAR to dephosphorylate β-catenin[52], β-catenin becomes activated to leave E-cadherin and results in the collapse of adherens junctions, allowing cells to migrate. As found in our study, the dissociation of β-catenin from E-cadherin causes E-cadherin ubiquitination and degradation[53], which might at least in part explains why S100A4 elevation is often found in association of E-cadherin loss, as shown in the study by Chen *et al*[2].

S100A4 in normal situation: An innocent bystander

As discussed above, S100A4 is expressed wherever cell migration is required, regardless normal or pathological situation. However, most of S100A4 studies focus on its bad side, such as cancer metastasis and organ fibrosis. Its good side has been continually overlooked. If we go back to the story that S100A4 was discovered in an experiment of serum stimulated fibroblasts, we know that S100A4 is innocent. Cells, including fibroblasts, in our body normally do not come into a direct contact with serum unless there is an injury. Therefore, when cells are suddenly exposed to serum, as the experiment done in Nathans' lab, they naturally interpret it as a signal of a wound. Therefore, a transcriptional program for wound healing gets activated immediately to battle against injury. S100A4 is just one of the players in this battle. So is SRF, and so are many SRF-regulated genes (e.g., C-FOS, EGR-1, CCN1, CTGF, FGF10, *etc.*)[54]. All these genes contain a common regulatory element CArG box, which SRF recognizes to bind. *S100A4* gene also contains such element in its promoter region[55], suggesting a possible regulation by SRF. *In vivo*, S100A4 activation has been found in various wound healings, and its contributions to tissue repair and modification are indisputable[16,56].

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FIELD OF VISION

NSAIDs for prevention of pancreatitis after endoscopic retrograde cholangiopancreatography: Ready for prime time?

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Abstract

Acute pancreatitis is the most common and the most fearful complication of endoscopic retrograde cholangiopancreatography (ERCP). Prevention of post-ERCP pancreatitis has therefore been of great interest to endoscopists performing ERCP procedures. So far, only pancreatic duct stenting during ERCP and rectal administration of a non-steroidal anti-inflammatory drug (NSAID) prior to or immediately after ERCP have been consistently shown to be effective for prevention of post-ERCP pancreatitis. This commentary focuses on a short discussion about the rates, mechanisms, and risk factors for post-ERCP pancreatitis, and effective means for its prevention with emphasis on the use of NSAIDs including a recent clinical trial published in *The New England Journal of Medicine* by Elmunzer *et al*[11].

INVITED COMMENTARY ON HOT ARTICLES

Acute pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP)[1,2]. It is important to distinguish between acute pancreatitis and hyperenzymemia after ERCP. Hyperenzymemia, defined as asymptomatic elevation of serum levels of amylase and lipase, is estimated to occur in more than 75% of patients undergoing ERCP and by itself does not have any clinical consequences[3]. Acute pancreatitis, on the other hand, is less common and can have significant clinical consequences. Although both conditions are characterized by elevation of serum levels of amylase and lipase, diagnosis of acute pancreatitis requires an additional factor, either pancreatic type pain or cross-sectional imaging confirming pancreatic inflammation[4].

Proposed underlying mechanisms that alone or in combination can induce post-ERCP pancreatitis (PEP) are prolonged manipulation around the papillary orifice causing edema, enzymatic injury from intestinal contents or contrast, hydrostatic injury from over-injection of the pancreatic duct, and thermal injury from electrocautery. There are probably other mechanisms involved that are yet to be recognized.

In most patients, the risk of PEP is in the range of 1%-10%. In high-risk cases, the risk can be as high as 30%[1]. Factors that convey a high risk for PEP can be

classified as patient-related, procedure-related, operator-related, and disease- or indication-related. Patient-related factors associated with a higher risk of PEP are younger age, female gender, and a normal serum bilirubin level. Procedure-related factors that have been suggested to be related to a higher risk of PEP include difficult cannulation, balloon dilatation of the biliary sphincter, and injection of contrast into the pancreatic duct particularly when acinarization occurs. Operator-related factors include lack of a good technique, lack of experience, and low case volume. The disease or indication for the ERCP is also important. For example, while the risk of PEP in patients undergoing ERCP for chronic calcific pancreatitis is very low, nearly one in 3 patients with type 3 sphincter of Oddi dysfunction undergoing ERCP will develop PEP.

Acute pancreatitis after ERCP is not a uniform disorder and varies in intensity[1,5]. Most cases of PEP are mild and resolve with proper treatment without any permanent sequela. A minority of the cases, however, is severe. Severe PEP is a feared complication of ERCP and can result in significant morbidity and mortality. Prevention of PEP has therefore been of major interest to endoscopists and significant time and effort have been devoted to finding endoscopic or pharmacologic means of preventing PEP.

So far, only pancreatic duct stenting and use of non-steroidal anti-inflammatory drugs (NSAIDs) consistently have been shown to be effective for PEP prophylaxis.

The first randomized trials assessing pancreatic duct stenting at the time of ERCP for PEP prevention were conducted in the 1990s[1]. Subsequent studies confirmed the effectiveness of this approach in decreasing the rate and severity of PEP, especially in high-risk patients.

Use of NSAIDs for PEP prophylaxis is relatively new. The rationale of NSAIDs administration for PEP prevention lies in their ability to inhibit substances such as prostaglandins, phospholipase A2 and neutrophil-endothelial interaction, which are believed to play an important role in severe inflammatory processes including acute pancreatitis[6]. The first clinical trial assessing the efficacy of a rectally administered NSAID for PEP prevention was reported in 2003 by a British group[7]. In that study, pancreatitis occurred in 6.4% of patients in the NSAID group compared to 15.5% in the placebo group. Two subsequent clinical trials by two independent Iranian research teams found that rectally administered NSAIDs were effective for PEP prevention[8,9]. A Mexican study confirmed those results[10].

The most recent clinical trial on use of a rectally administered NSAID for prevention of post-ERCP pancreatitis was published a few weeks ago[11]. In this clinical trial, 602 patients at elevated risk for post-ERCP pancreatitis were randomly assigned to receive a single dose of rectal indomethacin or placebo immediately after ERCP. The incidence of post-ERCP pancreatitis was significantly reduced among those receiving rectal indomethacin (9.2%) compared to those in the placebo group (16.9%).

In conclusion, based on the current literature, two prevention modalities have proven effective for PEP prophylaxis: (1) endoscopic placement of a pancreatic duct stent during ERCP; and (2) rectally administered NSAID immediately before or after ERCP.

Endoscopic pancreatic duct stenting for PEP prophylaxis in high-risk patients is a well-accepted strategy and is being used as a routine practice in most ERCP centers.

Although still not adopted as a routine practice, there is enough evidence to support the routine use of NSAIDs for PEP prevention at least in high-risk patients.

Although use of endoscopic and pharmacological means such as pancreatic duct stenting or rectally administered NSAIDs can decrease the rate and severity of PEP, they cannot, and should not replace the common sense. The best strategy for prevention of post-ERCP pancreatitis has been and remains avoiding unnecessary procedures. Other strategies for PEP prophylaxis include proper training of the endoscopists and assistants; adequate case volume to maintain proficiency; avoiding repeated injection to the pancreatic duct if evaluation of the pancreatic duct is not required; and referral of high-risk cases to specialized ERCP centers.

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FIELD OF VISION

B cell depletion in treating primary biliary cirrhosis: Pros and cons

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Abstract

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease of unknown etiology that affects almost exclusively women. Ursodeoxycholic acid (UDCA) is currently the only approved drug by Food and Drug Administration for patients with PBC. Although the precise pathogenesis of PBC remains unclear, it has been postulated that many cell populations, including B cells, are involved in the ongoing inflammatory process, which implicates, not surprisingly, a potential therapeutic target of depleting B cell to treat this disorder. Rituximab is a chimeric anti-CD20 monoclonal antibody that has been approved for the treatment of lymphoma and some autoimmune diseases such as rheumatoid arthritis. Whether it is effective in the treatment of PBC has not been evaluated. Recently, Tsuda *et al*[1] demonstrated that B cell depletion with rituximab significantly reduced the number of anti-mitochondrial antibodies (AMA)-producing B cells, AMA titers, the plasma levels of immunoglobulins (IgA, IgM and IgG) as well as serum alkaline phosphatase, and it was well tolerated by all the treated patients with no serious adverse events. This observation provides a novel treatment option for the patients with PBC who have incomplete response to UDCA.

Key words: Primary biliary cirrhosis; Rituximab; B cell depletion; Anti-mitochondrial antibodies

INVITED COMMENTARY ON HOT ARTICLES

We read with interest the recently published paper by Tsuda *et al*[1] describing an open-label study of rituximab treatment in six patients with primary biliary cirrhosis (PBC) who had an incomplete response to ursodeoxycholic acid (UDCA). We believe this observation provides a novel treatment option for the patients with PBC who have incomplete response to UDCA and would recommend it to the readers.

PBC is a cholestatic liver disease characterized by serological findings of anti-mitochondrial antibodies (AMA) and pathological non-suppurative destruction of biliary epithelial cells[2,3]. PBC may lead to liver failure or

even death. However, UDCA is the only Food and Drug Administration-approved drug and its efficacy is far from satisfaction in a large proportion of patients[4]. Recent studies have demonstrated that B cells are involved in immune mechanisms of the pathogenesis of non-suppurative cholangitis and the destruction of bile ducts in PBC[5-7]. These findings implicate a potential treatment efficacy of B cell depletion in patients with PBC[8-10].

Rituximab is a mouse-human chimeric anti-CD20 monoclonal antibody designed for B cell depletion in human. Its safety and efficacy as a single therapeutic agent has been demonstrated initially in the treatment of non-Hodgkin B cell lymphoma and chronic lymphocytic leukemia[11,12]. In addition, there were also clinical trials demonstrating that rituximab significantly induced clinical remission in a number of autoimmune diseases such as granulomatosis with polyangiitis, microscopic polyangiitis, and rheumatoid arthritis (RA)[13-15].

In the field of PBC, there were several studies in murine models investigating the treatment effect of B-cell depletion. Dhirapong *et al*[8] reported that B cell-depleted mice developed more aggressive PBC-like liver disease with increased infiltration of inflammatory cells around the damaged bile canaliculi in portal areas. Whereas Moritoki *et al*[16] showed that anti-CD20 therapy had no effect on adult dominant-negative transforming growth factor (TGF)-βR II mice (age range: 20-22 wk to 36-38 wk), and it neither alleviated liver inflammation nor exacerbated colitis. But in younger dominant-negative TGF-βR II mice aged 4-6 wk, anti-CD20 treatment significantly alleviated the liver inflammation and reduced the bile duct damage, suggesting that anti-CD20 treatment might be beneficial for patients with PBC of early disease stage.

Tsuda *et al*[1] used rituximab to treat six patients with PBC who had suboptimal biochemical response to UDCA. After B-cell depletion, they observed a reduction in the number of AMA-producing B cells, AMA titers, the plasma levels of immunoglobulins (IgA, IgM and IgG) as well as serum alkaline phosphatase (ALP) at week 24. As the levels of immunoglobulins, AMA titers and ALP returned to baseline levels at week 36, repeated anti-CD20 treatment was suggested to maintain the treatment effect. The necessity of repeated treatment with rituximab was also demonstrated by recent clinical trials on other autoimmune diseases such as RA and systemic lupus erythematosus, and this treatment strategy did not lead to permanent remission[17-19]. It is noteworthy that there was also study reporting that repeated treatment with rituximab could potentially compromise host protective immune response and might cause severe infection in RA patients[20]. In Tsuda's study on PBC patients[1], two patients (2/6, 33.3%) experienced reactivation of varicella zoster and upper respiratory infection after the first infusion of rituximab. Though it might be arbitrary to ascribe these infections exclusively to rituximab infusion, infections remain the major concern when treating patients with anti-CD20 antibodies. In PBC and other autoimmune diseases, it remains controversial if repeated anti-CD20 treatment is beneficial in terms of safety and efficacy, and if so, when is the optimal time for repeated therapy.

A high titer of serum AMA can be detected in 83%-95% of patients with PBC[21]. Most studies have shown that there is no correlation between the level of serum AMA and the severity of PBC, and AMAs positivity does not predict the patient's response to treatment with UDCA[22-25]. However, there were also some studies suggesting that AMA-positive PBC patients had more severe bile duct destruction than PBC patients with negative AMA[26]. AMAs could induce the caspase activation of the biliary epithelial cells and subsequent cell death and bile duct damage[27]. Tsuda *et al*[1] found that in the PBC patients, together with the number of peripheral B cells, the plasma levels of immunoglobulins and ALP, the level of AMA also decreased after treatment with rituximab and returned to baseline levels 36 wk after cessation of rituximab. They suggested that the depletion of the AMA-secreting plasma cells by rituximab could potentially reduce hyperactive B cell immune response and lead to the amelioration of the bile duct destruction in PBC, even though it is too early to jump to the conclusion that the level of serologic AMAs is a predicting factor for the efficacy of rituximab therapy.

Although B cell is one of the pivotal inflammatory cells in the immunopathogenesis of PBC, its precise role and the adverse events associated with B cell-depletion remain unclear[28]. A study reported that the morbidity of severe side effects of B cell-depletion is low but not insignificant[29]. There were also studies reporting new onset cases of inflammatory bowel disease that may be attributed to the B cell depletion in up to 40% patients with PBC[30,31]. In dominant negative TGF-βR II mice, Moritoki *et al*[16] found that anti-CD20 treatment induced up-regulation of interleukin 6, which could lead to exacerbation of colitis. Paradoxically, in some studies on murine models, B cells might play a protective role in PBC and B cell depletion exacerbated the biliary pathology and caused more aggressive PBC-like liver diseases[8,26,28]. There was also a case report showing that, after rituximab treatment, PBC developed with a high AMAs titer, intrahepatic cholestasis and steatorrhea in a RA patient[32], though it is not exactly understood if PBC was caused by immuno-mechanism underlying RA or by rituximab itself. In Tsuda's study on PBC patients[1], however, there was no evaluation of inflammatory bowel diseases and biliary pathology during follow-up. It should also be noted that, in their study, the number of enrolled patients and the duration of follow-up were not enough and the level of other biochemical parameters and PBC-40 scores remained unaltered. The long-term efficacy and prognosis could be the most important concern of rituximab treatment.

In conclusion, the study by Tsuda *et al*[1] suggests that B cell depletion with rituximab is potentially a promising treatment regimen for the PBC patients who do not have good response to UDCA. B cell depletion merits further investigation in human PBC to illuminate its safety and efficacy.

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Challenges of incorporating gene expression data to predict HCC prognosis in the age of systems biology

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Abstract

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide. The recurrence of HCC after curative treatments is currently a major hurdle. Identification of subsets of patients with distinct prognosis provides an opportunity to tailor therapeutic approaches as well as to select the patients with specific sub-phenotypes for targeted therapy. Thus, the development of gene expression profiles to improve the prediction of HCC prognosis is important for HCC management. Although several gene signatures have been evaluated for the prediction of HCC prognosis, there is no consensus on the predictive power of these signatures. Using systematic approaches to evaluate these signatures and combine them with clinicopathologic information may provide more accurate prediction of HCC prognosis. Recently, Villanueva *et al*[13] developed a composite prognostic model incorporating gene expression patterns in both tumor and adjacent tissues to predict HCC recurrence. In this commentary, we summarize the current progress in using gene signatures to predict HCC prognosis, and discuss the importance, existing issues and future research directions in this field.

INVITED COMMENTARY ON HOT ARTICLES

Hepatocellular carcinoma (HCC) is the sixth most common cancer type and the third leading cause of cancer-related death worldwide[1]. The major risk factor of HCC is chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV)[2]. So far, curative treatments for HCC include orthotopic liver transplantation, surgical resection and percutaneous ablation. However, the recurrence rates remain high and long-term survival is poor.

There are two types of HCC recurrence: early recurrence and late recurrence with different mechanisms. Early recurrence (< 2 years after the treatment) is mostly caused by metastasis and dissemination of primary HCC; while late recurrence ($\geq$ 2 years after the treatment) mainly

results from *de novo* tumors, as a consequence of field effect in diseased liver which is closely associated with high viral loads and hepatic inflammatory activities[3,4]. The treatment after curative therapy varies greatly, depending on individual's profile[5]. The traditional prognostic markers of HCC include vascular invasion (both macroscopic and microscopic) which is the most significant factor, tumor size, number of nodules, α-fetoprotein level, degree of differentiation, and satellites[6]. Recent advancement in the field has shown that viral factors and inflammation-related conditions are apparently associated with HCC prognosis. Viral load, genotype C, viral mutations, and expression of inflammatory molecules in HBV-related HCC tissues are significantly associated with poor prognosis. Host-inflammation-related factors such as imbalance between intratumoral CD8^{+} T lymphocytes and regulatory T lymphocytes, T helper (Th)1 and Th2 cytokines in peritumoral tissues are also predictors of HBV-related HCC[7,8]. In addition, non-coding RNA also plays a significant role in HCC progression[9]. However, even after incorporating viral and other factors, the prediction power can not be optimized. Therefore, it is crucial to identify new prognostic markers to better approach opportunities for individualized therapeutics for HCC patients.

The application of high-throughput methods has provided new opportunities for analyzing the diversity and heterogeneity of cancers. Studies of microarray-based gene expression profiling in breast cancer have shown a great success and led to a working model for a breast cancer molecular taxonomy[10]. Gene expression signatures suceeded in prognosis prediction and treatment responses for HCC[11], and they are promising in developing personalized cancer medication[12]. Gene expression profiles may add new and important prognostic information beyond those provided by the standard clinical predictors. It is important to incorporate molecular information to more accurately predict early and overall recurrence of HCC.

We read with great interest the recent article by Villanueva *et al*[13]. In this article, the authors developed an integrated prognostic model combining genomic and clinicopathologic data to improve outcome prediction in single-nodule early HCC patients. They analyzed the prognostic power of 22 previously reported gene signatures in a cohort of 287 early-stage HCC patients. The analysis showed that the proliferation signature was the most prevalent prediction (number of patients identified with the signature/number of total patients); and there was a substantial association among three groups of signatures: (1) signatures related to increased cell proliferation, progression in cell cycle and activation of specific pathways; (2) signatures generated in the adjacent tissues; and (3) cytokeratin-19 gene signature. They found that G3 (tumoral) signature and poor-survival (non-tumoral) signature, along with satellites were independent predictors of early tumor recurrence and overall recurrence. They also reported that genomic profiles of tumor and adjacent tissues were complementary in refining the prediction.

Advanced imaging techniques such as computed to-

mography and magnetic resonance imaging have been used to detect vascular invasion and conduct satellite evaluation before surgery, which are helpful in the pre-operative prediction of HCC prognosis. Genomic profiling using tumor and adjacent tissues obtained by fine-needle biopsy may provide complementary and/or confirmative information, thus having a great potential when combined with imaging findings in the clinical practice. Many studies have used array-based gene expression profiling obtained from tumoral or non-tumoral tissues to predict HCC prognosis. However, the number and heterogeneity of the signatures hinders their further application. The study of Villanueva *et al*[13] attempted to address these issues. They evaluated the prognostic predictive power of previously reported gene signatures in an independent cohort, and then developed a "composite genomic-based prognostic model". They further validated the stability of the model using samples from different sites of the same tumor nodule to test whether the genomic signature was consistent throughout different sites of a tumor[13]. This study presents a unified approach to systematically evaluate and independently validate HCC prognostic gene signatures; and the procedure developed in this study is conducive to the future studies of other complex disease.

Cancer gene signatures may indicate specific biological traits of heterogeneous tumor sub-phenotypes that cannot be identified by traditional methods. They may be associated with tumor biology and tumor microenvironment such as chromosomal instability, wounded stroma, or invasiveness, and possibly also linked to certain signaling pathways[14]. Gene signatures may have functional implications and may be predictive of response to specific therapeutic agents such as antiviral medications. Signatures identified in the study of Villanueva *et al*[13] (tumoral G3-proliferation signature and nontumoral poor-prognosis signature) reflect highly relevant biological events for outcome prediction and point out possible pathways to search for biomarkers as therapeutic targets. If used appropriately, gene signatures should be important complementary methods to current clinicopathological risk stratification systems[15]. Integrating gene signatures in HCC prognosis prediction may potentially improve patient outcomes, obtain a better understanding of the underlying HCC biology, and identify effective therapeutic options for an individual patient.

HCC is not a single disease at the molecular level. Using gene signatures to classify HCC into molecular subtypes with similar prognostic implication can guide clinical decision-making, particularly regarding therapy. However, these signatures lack prognostic power. The assignment of a given patient to a subgroup is strongly dependent on the gene signature used and the results from studies of a specific/single gene signature cannot necessarily be generalized. Furthermore, there are few genes overlapped among gene expression signatures which reflect common cellular phenotypes and yield similar predictions. Therefore, it is not appropriate to use overlapping in gene identity to measure the reproducibility of gene-expression profiles[16]. Thus, systematic evaluation of different gene

expression datasets and validation in independent cohorts provide basis for identifying true genomic signatures that are associated with oncogenic pathway, tumor biology and its microenvironment. Nevertheless, there are problems of using gene signatures to classify sub-phenotypes and predict HCC prognosis. In the following section, we take the paper of Villanueva *et al*[13] for an example to discuss several imposing issues in the field.

First, the paper does not mention whether evaluation on the quality of the different gene signatures was used. These signatures were generated from different samples with different biological background. Different studies may vary greatly in study quality, such as patient selection criteria, RNA quality, follow-up criteria, definition of prognosis, treatment after surgery, *etc*. Patient differences including different staging and underlying conditions may reflect etiological differences, thus resulting in the heterogeneity of gene signatures. Prognostic accuracy might differ in tumors with different stages. Additionally, multiple end points, such as overall recurrence, early recurrence, late recurrence, overall survival, or metastasis-free survival, used in the analyses are also the source of heterogeneity. There is also the possibility of stromal contamination, namely, gene signatures derived from analysis of tumor specimens with a high proportion of adjacent tissue contamination, and vice versa. The general reproducibility of these signatures stands out as an important issue.

Second, it is inappropriate to directly combine datasets from different platforms and different experiments because of the non-biological experimental variation or batch effects. In the study of Villanueva *et al*[13], gene expression data were obtained from 3 high-throughput genomic platforms, and these datasets cannot be readily put together because of their heterogeneity. Again, the authors did not mention whether any standardization procedures were applied. In addition, the method used for integration and/or standardization of different platforms is also a challenge. How to choose a robust normalization method according to the features of the dataset to reduce the batch effect is essential for further computational analyses[17].

Third, the authors did not describe whether they applied the gene mapping procedure. Gene database updates with time, with the accumulation of information, the platform used several years ago may not be comparable to the gene database in service now. Without mapping, the genes in the 22 signatures produced at different time points may not correspond well. Accurately mapping and matching a gene across different signatures generated by different platforms at different time points is an important quality control step to enable the finding of true signatures.

Last but not least, the quality of survival analyses used to generate these signatures differs. The frequently used statistical methods, such as the significant analysis of microarray tool, the trend filter tool, and Cox's proportional Hazard model, may contribute to the great variety of gene expression signatures[17]. Different studies also vary in terms of follow-up information collected, covariates adjusted in multivariate analysis, and non-informative censoring. These directly affect the gene signatures generated.

For gene signatures to be used in clinical practice to accurately predict HCC prognosis, the following procedures are required. For a start, there should be a standardization of tissue composition. Without appropriate and standardized samples, the further experiments to determine a robust signature will be difficult. For example, the variable selection procedure is crucial in developing reliable and reproducible gene signature because preanalytical variables such as stromal component and tissue processing will directly affect gene expression profiles. In addition, to enable the usage of data by different researchers and future investigators, a detailed description of data processing and analytical methods is required. A further step is to establish unified high criteria for generating gene expression signatures. Moreover, it is also important to identify gene signatures to predict early and late recurrence of HCC. HCCs are a group of diverse and heterogeneous diseases. Gene expression patterns can provide a basis to distinguish sub-phenotypes within the heterogeneity subgroups characterized by conventional clinicopathological variables, and also present important information about individualization of therapy[18]. Viral mutations in the preS and the basal core promoter regions of HBV are significantly associated with HCC risk[19-23]. The HBV mutations including A1762T/G1764A, preS deletion at nt.107-141, and preS2 mutations in adjacent hepatic tissues and the HCV mutation such as M91L are significantly associated with poor prognosis of HCC[24-26]. The viral mutations should be reasonably integrated into the HCC prognosis-related gene signature.

To summarize, this paper drew our interests because gene expression signatures have shown great promise in classifying cancer subtypes and predicting prognosis. The Villanueva team has introduced an effective approach to systematically integrate different types of data for HCC prognosis prediction. With the increasing amount of data produced, there is an urgent need of standardized methods in systems biology to integrate descriptive data from cohort studies and other sources such as clinicopathological features, massive DNA and RNA parallel sequencing, and proteomics, along with functional data to guide therapeutic decisions. In addition, data on vascular features of HCC from imaging techniques may help select and validate true gene expression signatures associated with HCC prognosis. Future studies should also correlate these two non-invasive and innovative methods. It is still premature to use the current gene signatures for predicting HCC prognosis in the context of clinical practice. There is enormous work to be done for these gene signatures to be used in routine clinical practice and treatment decision making.

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GUIDELINES FOR CLINICAL PRACTICE

Overview and developments in noninvasive diagnosis of nonalcoholic fatty liver disease

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Abstract

High prevalence of non-alcoholic fatty liver disease (NAFLD) and very diverse outcomes that are related to disease form and severity at presentation have made the search for noninvasive diagnostic tools in NAFLD one of the areas with most intense development in hepatology today. Various methods have been investigated in the recent years, including imaging methods like ultrasound and magnetic resonance imaging, different forms of liver stiffness measurement, various biomarkers of necroinflammatory processes (acute phase reactants, cytokines, markers of apoptosis), hyaluronic acid and other biomarkers of liver fibrosis. Multicomponent tests, scoring systems and diagnostic panels were also developed with the purposes of differentiating non-alcoholic steatohepatitis from simple steatosis or discriminating between various fibrosis stages. In all of the cases, performance of noninvasive methods was compared with liver biopsy, which is still considered to be a gold standard in diagnosis, but is by itself far from a perfect comparative measure. We present here the overview of the published data on various noninvasive diagnostic tools, some of which appear to be very promising, and we address as well some of still unresolved issues in this interesting field.

INTRODUCTION

Finding a means to noninvasive diagnosis of non-alcoholic fatty liver disease (NAFLD) and its entities has been the aim of many research efforts since recently, and seems to remain a very much needed goal among many clinicians and researchers in the field of hepatology. Why is it that way?

NAFLD is today considered to be the most common liver disease in adults. The prevalence of NAFLD in general population is very high, in the range of 15%-30% according to various studies, and is even increasing, due to

the rising prevalence of diabetes and obesity[1]. Spectrum of NAFLD includes two entities with very different natural course and prognosis: simple steatosis, which mostly has a benign non-progressive course and good prognosis, and non-alcoholic steatohepatitis (NASH), which demonstrates progression of fibrosis in about 30%-40% of patients and has a proven potential to eventually lead to cirrhosis and end-stage liver disease including hepatocellular carcinoma[2-4]. NASH seems to be present in a surprisingly high proportion of NAFLD patients, including 40% to 75% of cases with elevated aminotransferase levels, those data coming from recent studies using current histological definitions and including substantial number of patients[2,5,6]. In studies of liver biopsy findings from apparently healthy living liver donor candidates, the proportion of NASH among patients with newly discovered NAFLD was about 30%[7]. Even in patients with normal aminotransferase levels, proportion of NASH among NAFLD cases seems to be almost the same, and the whole spectrum of NAFLD including advanced fibrosis and cirrhosis has been observed in patients with completely normal laboratory findings[6,8,9].

Liver biopsy is still considered the gold standard in diagnosis and the only reliable tool for distinguishing NASH from simple steatosis and for grading and staging the disease, providing important information about severity of steatosis, lobular inflammation, hepatocellular ballooning, and degree of fibrosis[10]. Minimal histological criteria for NASH include steatosis, hepatocyte injury (in the form of ballooning or apoptosis) and lobular inflammation. Similarly to other chronic liver diseases, fibrosis is usually divided histologically in four stages: perisinusoidal fibrosis (F1), perisinusoidal and periportal fibrosis (F2), bridging fibrosis (F3) and cirrhosis (F4). Liver biopsy also has several negative aspects: it is invasive, unpleasant for patients, it usually includes hospitalization and a day or two lost at work, and the adequate interpretation of the specimen requires a pathologist with expertise in hepatopathology, which altogether makes it a costly and time-consuming procedure. Another significant drawback of liver biopsy, and in medical terms the most important one is its substantial sampling variability, which has been consistently proven for several chronic liver diseases including NAFLD. In a well-designed study by Ratziu *et al*[11], the negative predictive value of a single biopsy for the diagnosis of NASH was calculated to be only 74%.

Considering the mentioned high prevalence of NAFLD in the general population, and the fact that every patient with NAFLD including the one with normal aminotransferases can potentially have NASH, we come to the conclusion that it would be necessary to perform liver biopsy in about one fourth of the whole Western population. This is clearly not feasible, but is it necessary? Until recently, many have advocated against the routine use of liver biopsy for patients with NAFLD because fatty liver is still considered by many to be a benign condition-although many studies have now clearly indicated a progressive course in a proportion of patients with NASH. Another reason for avoiding biopsy and definite diagno-

sis was the lack of established pharmacological treatment options which would have proven efficacy in preventing progression or leading to regression of disease.

Although there are still no generally approved treatments for NASH, several treatment options have demonstrated efficacy in various clinical trials, and for example recently published results of the randomized multicenter pioglitazone *vs* vitamin E *vs* placebo for the treatment of nondiabetic patients with nonalcoholic steatohepatitis (PIVENS) trial have provided substantial evidence for the previously suggested efficacy of vitamin E (and to a lesser extent pioglitazone) in inducing histologic improvement of NASH[12]. Hopefully, we could soon expect to have several efficient treatment options available. All of this pretty much eliminates the validity of approach where NASH remains undiagnosed, which currently does happen in many clinical settings, e.g., in patients with accidental ultrasonographic finding of fatty liver and even patients with mildly elevated transaminase levels and known NAFLD risk factors who are very often not investigated any further.

The necessity of diagnosing NASH and the proportion of the population affected lead to a logical conclusion that a need for a reliable noninvasive tool in NAFLD diagnosis is highly urgent. Ideal noninvasive tool would be able to distinguish NASH from simple steatosis and allow for grading and staging of disease, which would largely facilitate screening of population at risk. Development of noninvasive tools would also enable monitoring of disease course and progression and evaluation of response to therapy, both in routine practice and in the setting of clinical trials, which is currently only possible with a follow-up liver biopsy. Another very important, and somewhat disregarded point, is that an efficient biomarker or set of biomarkers would accurately reflect the inflammatory and fibrotic processes on the level of the whole of liver parenchyma, thereby increasing the diagnostic accuracy and resolving the problem of sampling variability intrinsic to liver biopsy, which represents only about 1/50.000 part of the organ which is not homogeneously affected by disease features.

In the text below, we present the current level of knowledge and progress regarding the noninvasive diagnostic tools that have been studied in the context of NAFLD (Table 1).

ROUTINE LABORATORY TESTS

Patients with NAFLD are mostly asymptomatic and the disease is usually suspected based on either hyperechoic liver appearance on abdominal ultrasound or mild to moderate increases in liver enzyme levels. These are usually the only aberrations that can be encountered in this patient population (apart from signs of associated conditions like elevated glucose or lipid levels), and a large proportion of patients has completely normal laboratory findings. Hypoalbuminemia, prolonged prothrombin time, and hyperbilirubinemia are parameters of impaired liver function and occur only in patients who have already de-

Table 1 Overview of noninvasive methods in diagnosis of liver disease severity that have been evaluated in the context of non-alcoholic fatty liver disease

Routine laboratory tests
  Liver enzymes
  Parameters of liver dysfunction
Imaging methods
  Ultrasound
  Computed tomography
  Magnetic resonance imaging
  Magnetic resonance elastography
Liver stiffness measurement
  Transient elastography (FibroScan)
  Acoustic radiation force impulse shear wave imaging
Biomarkers of necroinflammation
  Cytokeratin 18 fragments
  High-sensitivity C-reactive protein
  Interleukin-6
  C-C chemokine ligand 2
  Plasma pentraxin 3
  Oxidative stress measurement
  Tumor necrosis factor-α
  Adiponectin
  Insulin resistance measurement
Multicomponent tests for diagnosis of non-alcoholic steatohepatitis
  Nash test
  Non-alcoholic steatohepatitis clinical scoring system for morbid obesity
  Model by Miele et al[61]
Biomarkers of fibrosis
  Hyaluronic acid
  Laminin
  Type VI collagen 7S domain
Multicomponent panels for diagnosis of fibrosis
  Fibrotest
  Non-alcoholic fatty liver disease fibrosis score
  European liver fibrosis panel/enhanced liver fibrosis panel

veloped cirrhosis. Most commonly elevated enzymes are alanine aminotransferase (ALT) and γ-glutamyltransferase (GGT), while aspartate aminotransferase (AST) elevation is less frequent and when pronounced may indicate presence of advanced fibrosis[13]. Many studies have tried to correlate liver enzyme levels with histological severity and progression of disease, and various results have been obtained. In some cases ALT, in other AST or GGT levels demonstrated best correlation with severity of inflammation or fibrosis and their progression/regression on follow-up biopsies[4,14-16]. Equally important, the full spectrum of NAFLD including severe inflammation and fibrosis was proven to occur with almost similar frequency in patients with completely normal liver enzymes[8,9].

IMAGING METHODS

Imaging modalities frequently used in the diagnosis of NAFLD include ultrasound, computed tomography and magnetic resonance imaging (MRI). While they are all very sensitive (80%-100%) and specific in detection of steatosis, none of them can effectively distinguish simple steatosis from NASH or determine the degree of fibrosis[17]. Nevertheless, MRI is more sensitive than ultrasound in detecting lesser degrees of hepatic steatosis, and new techniques in MRI are constantly being developed that

provide additional data on different tissue parameters. One of them is magnetic resonance (MR) elastography, which estimates liver tissue stiffness by imaging the propagation of induced shear waves with a modified phase-contrast MR sequence. This technique was shown to have an excellent predictive value for excluding fibrosis, while sensitivity and specificity for discriminating between mild and more severe fibrosis was around 85%[18]. A recent study investigated the performance of MR elastography in 58 patients with NAFLD and demonstrated very high accuracy with under the receiver operated curve (AUROC) of 0.93 for discriminating patients with NASH and those with simple steatosis, with a sensitivity of 94% and a specificity 73% by using a threshold of 2.74 kPa[19]. The future advances in MRI technology including hepatic flow parameters and diffusion-weighted MRI may hopefully provide more MR-based tools for liver fibrosis detection. Ultrasound has also demonstrated the potential for improvement in diagnosis of NAFLD and NASH. Apart from ultrasound-based elastography, use of ultrasound contrast agents has been studied in this scenario, and signal intensity after contrast administration was shown to be significantly lower in NASH when compared with simple steatosis and normal liver[20].

LIVER STIFFNESS MEASUREMENT

Transient elastography (FibroScan®, EchoSens, Paris, France) is a relatively novel technique which measures liver tissue elasticity by measuring the speed of propagation of probe-induced vibrations through parenchyma by ultrasound. Elasticity shows significant correlation with degree of liver fibrosis, and FibroScan is considered to produce a reliable prediction of higher degrees of liver fibrosis. The method was first assessed in population of patients with hepatitis C, and after evaluation in multiple studies it has been introduced into clinical practice. A survey performed four years ago in France showed that about a third of hepatologists was using it (mostly in evaluation of patients with hepatitis C), and the method is now gaining increasing popularity in other countries as well[21]. More recent studies have assessed transient elastography in population of patients with NAFLD, and obtained results that are similar to those from studies in hepatitis C[22-24]. A consistent increase in liver stiffness with increasing fibrosis stage was observed, and the largest study, performed by Wong et al[22], obtained AUROC values of 0.93 for advanced fibrosis and 0.95 for cirrhosis. When the liver stiffness cut-off was set at 7.9 kPa, negative predictive value for advanced fibrosis was excellent (97%), and could be applied to 60% of the population. On the other side, positive predictive value of having advanced fibrosis or cirrhosis was at best only 72.4%, at the 9.6 kPa cut-off. Accuracy of FibroScan in detecting significant fibrosis (defined as at least perisinusoidal and portal/periportal fibrosis) was poor, as was expected from previous experience.

Several meta-analyses have assessed performance of transient elastography in fibrosis detection, consisting

mostly of studies on hepatitis C patients. They have shown generally very good diagnostic accuracy in detecting cirrhosis and somewhat lesser precision in excluding advanced fibrosis, while they demonstrated substantial heterogeneity in diagnosis of significant fibrosis ($F \geq 2$)[25,26]. Importantly, variation in cut-off values of liver stiffness has been large and these values still require validation. In conclusion, due to the relatively low specificity, the value of transient elastography seems to remain in ruling out cirrhosis and advanced fibrosis in patients with low liver stiffness values, while patients with intermediate values would still require liver biopsy for correct classification, and the proportion of patients with high stiffness values who are misclassified is not negligible. It is also important to take into account that the population of patients that is usually encountered in clinical practice does not have advanced fibrosis or cirrhosis in large proportions, and diagnosis of lesser degrees of fibrosis is equally important in estimating the risk for liver-related morbidity and mortality. A large study that evaluated frequency and reasons of failure to obtain the elasticity measurement found that FibroScan was feasible in over 95% of the patients, and the only factor associated with failure was body mass index greater than 28[27]. Failure occurs due to the elastic and ultrasound wave attenuation by subcutaneous fat, and while this may not be a significant issue in other chronic liver diseases, it is an important limitation in patients with NAFLD, considering the prevalence of obesity in this population. In the study by Wong *et al*[22], measurement could not be obtained in over 10% of cases, which significantly reduced diagnostic accuracy when the 'intention-to-diagnose' analysis was performed.

Another noninvasive method of assessing tissue stiffness, acoustic radiation force impulse (ARFI) shear wave imaging, was recently assessed in a couple of studies with NAFLD patients[28,29]. This ultrasound-based technique estimates the tissue stiffness by measuring transient tissue deformations of several microns which are induced in the liver parenchyma by acoustic radiation force. In a study on 172 NAFLD patients by Palmeri *et al*[29], ARFI imaging distinguished low (Stages 0-2) from high (Stage 3-4) fibrosis stages with a sensitivity and a specificity of around 90% (AUROC of 0.90). Body mass index over 40 kg/m^2 was not a limiting factor for ARFI imaging, which overcomes part of the problems associated with FibroScan. When compared to FibroScan, ARFI imaging demonstrated similar diagnostic performance[28,30].

BIOMARKERS OF NECROINFLAMMATION

Most intense research is now being focused on biomarkers, measurable serum parameters that reflect the intensity of inflammatory processes and hepatocyte necrosis, as well as the ones that reflect extracellular matrix remodelling and collagen deposition. Ideally, an excellent biomarker would be specific for liver and accurately reflect the underlying pathogenetic processes on the level of whole organ, and thus be an even more precise indicator of the

disease than liver biopsy, which is prone to sampling variability and interpretation biases as described earlier.

Cytokeratin 18 fragments

Apoptosis is an important mechanism in pathogenesis of NASH, and its initiation leads to activation of caspase family of intracellular proteases which then cleave different intracellular proteins including cytokeratin 18 (CK-18), the major intermediate filament protein in hepatocytes. By measurement of CK-18 fragments hepatocyte apoptosis can be quantified, and this method was tested as a noninvasive tool in NASH diagnosis in several studies. Initial results were very promising, as Wieckowska *et al*[31] demonstrated a striking increase in serum CK-18 fragment levels in patients with definitive NASH, as well as their high diagnostic accuracy for differentiating between NASH and simple steatosis or normal liver, with AUROC of 0.93 and positive and negative predictive value of 95.0% and 89.5%, respectively. However, this study included only 39 patients, and the larger validation study that was subsequently undertaken and included 139 patients obtained less favourable results: median CK-18 fragment levels in NASH cases were now only 335 U/L (compared to 765 U/L in the first study, and to about 200 U/L in non-NASH cases in both studies), and diagnostic performance was expectedly poorer (calculated AUROC was 0.83 and sensitivity for diagnosing NASH was at best 77%, with the specificity rising above 90% only at the highest tested cut-off value)[32]. Nevertheless, CK-18 fragment levels showed very good correlation with NASH, fibrosis and NAS (NAFLD activity score), and similar results were reported from other groups as well, supporting its potential role as a noninvasive tool in NAFLD[33-35]. Even more importantly, in the study by Diab *et al*[35,36] on 99 patients who underwent bariatric surgery, CK-18 fragment levels showed a significant decrease 6 mo postoperatively, and in another study changes in CK-18 fragment levels closely paralleled changes in NAS on follow-up biopsy. These findings indicate the potential use of CK-18 fragment levels in the follow-up of patients with NASH, including evaluation of response to therapy. This certainly requires further attention, as it could possibly lead us closer to the goal of eliminating the need for second liver biopsy and thus facilitating design and conductance of clinical trials, as well as enhancing patient follow-up in clinical practice.

High-sensitivity C-reactive protein

C-reactive protein (CRP) is an acute-phase reactant produced by the liver in many inflammatory conditions, and based on the hypothesis that NASH is associated with low-grade systemic inflammation, several studies have compared high-sensitivity CRP levels in patients with NAFLD. Two studies found that hs-CRP levels were significantly higher in cases with NASH compared to those with simple steatosis, and hs-CRP also correlated well with presence of advanced fibrosis[37,38]. However, a study performed earlier concluded that measurement of hs-CRP

was not useful in predicting the histological severity of NAFLD, as there was no relationship between the levels of hs-CRP and the grades of steatosis, necroinflammation or fibrosis[39]. Further investigation including testing of diagnostic accuracy is needed before definite conclusions can be reached about usefulness of this marker in NAFLD.

Interleukin-6 and C-C chemokine ligand 2

Interleukin-6 (IL-6) is a proinflammatory cytokine that is involved in NAFLD pathogenesis, and Wieckowska *et al*[40] demonstrated a markedly increased IL-6 expression in liver tissue of patients with NASH as compared to simple steatosis or normal liver, with a positive correlation with severity of inflammation and fibrosis. Plasma IL-6 levels that were parallely measured in this study correlated well with liver IL-6 expression. In another study, IL-6 was among several serum markers evaluated in 47 NAFLD patients and 30 controls, and it was significantly increased in patients with NAFLD as compared to controls, but not in NASH compared to simple steatosis[41]. This study also evaluated serum levels of C-C chemokine ligand 2 (CCL2), a chemokine responsible for monocyte/macrophage infiltration of liver and maintaining hepatic inflammation and fibrogenesis, and found that it was significantly elevated in patients with NASH compared to simple steatosis, but diagnostic performance of CCL2 levels was not tested. In a recent study, pharmacological inhibition of CCL2 had an effect on reduction of hepatic steatosis in a murine model, and CCL2 will presumably see some further investigation in the context of NAFLD[42].

Plasma pentraxin 3

Plasma pentraxin 3 is a novel marker of systemic inflammation from pentraxin family of acute-phase proteins that is produced by diverse cell types in response to proinflammatory cytokines[43]. Yoneda *et al*[44] have evaluated pentraxin 3 levels in 70 patients with NAFLD, and found that they were significantly higher in cases with NASH compared to non-NASH, with the AUROC value of 0.75 for NASH detection. Pentraxin 3 levels also correlated well with the stage of fibrosis. These findings should provide basis for additional evaluation of this marker in other NAFLD patient cohorts.

Oxidative stress

Oxidative stress is one of the key mechanisms in NASH pathogenesis, and several studies have measured systemic markers of oxidative stress status in NAFLD patients and compared them between cases with NASH and controls[45-47]. Different methods for measurement of oxidative stress have been used (measurement of levels of lipid peroxidation products, levels of antioxidant defence systems like vitamin E, glutathione peroxidase and superoxide dismutase activities, antioxidant capacity of the plasma and total plasma peroxide concentrations), and studies produced disparate results. Based on the current data, there is no doubt that oxidative stress is present in

NASH, but the utility of its measurement as a noninvasive tool in NAFLD diagnosis probably does not have any clinical value.

Tumor necrosis factor-α and adiponectin

Tumor necrosis factor (TNF)-α and adiponectin are cytokines which have been proven to play important roles in NAFLD pathogenesis, and the serum levels of these cytokines were determined in patients with NAFLD and correlated to disease severity in multiple studies[48-52]. However, diagnostic accuracy in discerning NASH from simple steatosis and the potential for noninvasive use in diagnosis were generally not evaluated, and the data on diagnostic performance of this cytokines are not available. As of the published results, most of the studies demonstrated correlation of lower adiponectin levels with presence of NAFLD compared to healthy controls, presence of NASH compared to simple steatosis, and with histological severity of the disease, while levels of TNF-α and its soluble receptor were most often not significantly different between patients with NASH and patients with simple steatosis or controls. Thus, the potential for clinical use of this cytokines as noninvasive tools for diagnosis of NASH is questionable.

Insulin resistance

Insulin resistance state leads to increased lipolysis and free fatty acid flux to the liver, and elevated plasma glucose and insulin levels promote *de novo* fatty acid synthesis and impair β-oxidation, contributing to the development of hepatic steatosis. Although it is not clear whether insulin resistance causes hepatic steatosis or the liver fat accumulation represents the primary event leading to peripheral insulin resistance, there is no doubt that it plays an important role in the pathogenesis of NAFLD. Large population studies have shown that almost all of the NAFLD patients were insulin resistant according to the homeostasis model assessment of insulin resistance (HOMA-IR)[53]. Additionally, the potential of insulin resistance measurement as a noninvasive diagnostic tool was also evaluated. In a study by Shimada *et al*[54], the authors tested the diagnostic performance of adiponectin, insulin resistance measured by HOMA-IR, and type IV collagen 7S in discriminating NASH from simple steatosis. While performance of each of these markers individually wasn't great, sensitivity of the combination of three markers was 94%, with a specificity of 74%.

Although insulin resistance has been usually associated with type 2 diabetes, it can also be present in type 1 diabetic patients[55]. The euglycemic insulin clamp technique which represents the gold standard for identifying type 1 diabetic patients who are insulin resistant is impractical for routine clinical use, and insulin resistance in type 1 diabetic patients was often recognized only by higher insulin requirements. Recent introduction of a validated method for estimated glucose disposal rate (eGDR) measurement based on clinical parameters has allowed its easier assessment in a clinical setting[56]. A recently pub-

lished study demonstrated that NAFLD markers were associated with insulin resistance measured by eGDR in type 1 diabetic patients. NAFLD associated markers (ALT, AST, alkaline phosphatase, GGT and ferritin) worsened in parallel with the decline in insulin sensitivity and after adjustment for covariates, ALT, AST and alkaline phosphatase were independent predictors of insulin resistance[57].

Multicomponent tests

There have been several attempts at constructing a panel of clinical and laboratory parameters that would, when combined using a formula or a scoring system, result in a value that enables distinguishing bewteen NASH and simple steatosis. The most advanced attempt was a study by the French group specialized at developing diagnostic models for various liver conditions, who constructed a complex test (NashTest) which combines 13 parameters (age, sex, height, weight, triglycerides, cholesterol, α2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST and bilirubin) into a patented algorithm[58]. Their design and validation study included 257 patients and 383 controls, and the NashTest had AUROC of 0.79 [95% confidence interval (CI) 0.69-0.86], with sensitivity for NASH (using criteria by Kleiner *et al*[59]) of only 33% and positive predictive value of 66%. The results were somewhat better when subgroups with borderline NASH and NASH were combined, the sensitivity rising to 88% and positive predictive value to 74%. In another study, a clinical scoring system was developed based on the results of multivariate analysis in a group of morbidly obese patients that underwent intraoperative liver biopsy at bariatric surgery[60]. The proposed NASH Clinical Scoring System for Morbid Obesity included 6 clinical variables (hypertension, diabetes, AST, ALT, sleep apnea and non-black race) and was used to stratify morbidly obese into 4 groups regarding the risk for presence of NASH (low, intermediate, high and very high). In the studied group, the proportion of patients with low-risk score who had NASH was 13%, while it was 80% in those with very high-risk score. Recently, Miele *et al*[61] measured several markers of liver fibrosis in a cohort of 46 patients with NAFLD, and constructed a mathematical model based on the results of multivariate analysis that included age, hyaluronic acid and tissue inhibitor of metalloproteinase 1 levels. A specific cut-off value identified patients with NASH with 86% sensitivity, and negative and positive predictive values of 96% and 60%. This model could potentially be useful in excluding patients with negative values from liver biopsy consideration if these findings are confirmed in larger independent studies.

BIOMARKERS OF FIBROSIS

As with other chronic liver diseases, the most important indicator of severity and progression of liver damage in NAFLD is the presence and degree of liver fibrosis. Estimation of fibrosis is therefore essential in the diagnostic workup of patients with NAFLD, and it remains one of the major reasons for performing liver biopsy in this population. After a large number of studies was undertaken in hepatitis C patients that tried either to design tests and scoring systems using combinations of readily available clinical and biochemical parameters, or to find specific biomarkers of fibrosis processes that would adequately correspond to liver biopsy findings, similar attempts were made as well in populations of patients with NAFLD. Generally, while showing good accuracy in detection of advanced fibrosis or cirrhosis, all of these tests demonstrate significantly lower sensitivities in predicting the presence of mild or moderate fibrosis. The problem lies in the fact that this is exactly the group of patients that would benefit most from therapeutic interventions, before significant fibrosis has already developed, and they therefore require early diagnosis.

Hyaluronic acid and other markers of extracellular matrix turnover

Hyaluronic acid is a component of the extracellular matrix that can be measured in serum, where it partially enters through lymphatics. Serum levels are dependent on production, which increases with increased collagen synthesis, as well as degradation, which takes course in liver sinusoidal endothelial cells after binding to specific receptors. With progression of liver fibrosis, both increased production of collagen and decreased function of sinusoidal endothelial cells lead to elevation of hyaluronic acid serum levels.

Several groups have so far evaluated the potential use of hyaluronic acid levels in diagnosis of NASH-related fibrosis. Suzuki *et al*[62] investigated the potential of hyaluronic acid for use in diagnosing fibrosis in a cohort of 79 patients with NAFLD and various degrees of fibrosis. The hyaluronic acid serum levels demonstrated good correlation with the degree of hepatic fibrosis, and significant difference was noted especially when comparing mild to moderate (Stages 0-2) with severe fibrosis or cirrhosis (Stages 3-4). The calculated AUROC for severe fibrosis was 0.89 (95% CI 0.81-0.97), and at the optimal cut-off value of 46.1 ng/mL sensitivity was 85% (95% CI 62%-97%) and specificity 80% (95% CI 67%-89%). When a prevalence of severe fibrosis among NAFLD patients was assumed to be 20% (approximate of usual patient population at referral centers), the corresponding positive predictive value was 51% (95% CI 39%-68%) and negative predictive value 95% (95% CI 91%-100%). Accuracy for diagnosing mild fibrosis (Stage 1) was low and the number of patients with moderate fibrosis (Stage 2) was inadequate for valid analysis. Another study evaluated hyaluronic acid and laminin levels in 50 patients with NASH, of whom 23 had some degree of fibrosis and 27 had no fibrosis on liver biopsy[63]. Subjects with NASH and fibrosis had significantly higher hyaluronic acid and laminin levels than those without fibrosis, and AUROC and diagnostic performance of both of these markers was calculated for differentiating between presence and

absence of fibrosis, showing excellent diagnostic accuracy of hyaluronic acid at the cut-off value of 148.8 ng/mL (reported sensitivity and specificity was over 95%). In the fibrosis group, levels of hyaluronic acid significantly increased with rising fibrosis stages, however the accuracy for distinguishing different fibrosis stages was not tested due to small patient numbers. Sakugawa *et al*[64] investigated the levels of hyaluronic acid and type VI collagen 7S domain in a population of 112 patients with NAFLD, of whom 70 were classified as NASH. On regression analysis, both markers were independently associated with the presence of NASH or severe fibrosis, but demonstrated sensitivity and specificity for severe fibrosis in the range of 70%-80%. However, if both markers were negative in a given patient, severe fibrosis was highly unlikely to be present (negative predictive value 95.2%).

Although some of these results look very promising, studies are still lacking in power, and the proposed cut-off values and calculated diagnostic accuracies are quite heterogeneous, which may be due to other factors in addition to difference in sample size, like difference in measurement methods and studied populations. Another important aspect of clinical usefulness of hyaluronic acid and other serum markers of fibrosis that hasn't yet been investigated is the question of sensitivity to longitudinal changes in fibrosis of liver parenchyma, which could potentially enable noninvasive patient follow-up and evaluation of treatment effects.

Multicomponent panels

FibroTest is a copyrighted panel developed by the French group who originally conceived it for diagnosis of liver fibrosis in hepatitis C, where it has subsequently been extensively studied. It includes 5 biochemical parameters (α2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and GGT) that are incorporated into a patented formula. More recently, the authors of the panel conducted a study that thoroughly evaluated diagnostic performance of FibroTest in the setting of NAFLD by including 267 patients and a large number of healthy controls[65]. Fibrosis stage was determined according to Kleiner *et al*[59], and advanced fibrosis included stages F2-F4 (perisinusoidal and portal/periportal fibrosis, bridging fibrosis and cirrhosis). Mean FibroTest value steadily increased with increasing fibrosis stage, and calculated AUROC for advanced fibrosis was 0.86 (95% CI 0.77-0.91), while it was 0.92 (95% CI 0.83-0.96) for bridging fibrosis or cirrhosis (F3-F4). A FibroTest cut-off score of 0.30 had 77% sensitivity and 90% negative predictive value, and score of 0.70 had 98% specificity and 76% positive predictive value for advanced fibrosis. As expected, performance was even better in detection of F3-F4, with 92% sensitivity and 98% negative predictive value. In addition to one third of patients having the value that fell between these two cut-offs and thus a nondiagnostic test result, other causes of FibroTest failure were analyzed and included Gilbert's syndrome, acute inflammation, and abnormal apolipoprotein A1 that was related to dyslipidaemia.

Another large multicenter study by a different group was undertaken and included a total of 733 patients divided in 2 groups in an attempt to develop and validate a noninvasive scoring system that would separate NAFLD patients with and without advanced liver fibrosis[66]. The score was named NAFLD fibrosis score and included a formula with 6 variables (age, hyperglycemia, body mass index, platelet count, albumin and AST/ALT ratio), selected based on results of multivariate analysis. Biopsy was also scored according to Kleiner *et al*[59], but the diagnostic goal of advanced fibrosis included only Stages F3 and F4. AUROC values were 0.88 in estimation and 0.82 in validation set, and two cut-off points were determined similarly to the previously mentioned study. Using the low cut-off point, negative predictive value of the score was 93% in estimation group and 88% in validation group, while with the high cut-off point positive predictive value was 90% in estimation group and 82% in validation group. Only 25% of patients had score values between the cut-offs and would thus be considered as "indeterminate" and still require liver biopsy after this noninvasive test was performed.

After the European Liver Fibrosis Group developed an algorithm that included age, tissue inhibitor of matrix metalloproteinase 1, hyaluronic acid and aminoterminal peptide of pro-collagen III and tested its performance in diagnosing significant fibrosis in a large cohort of patients with various chronic liver diseases, another study was undertaken more recently that investigated performance of this panel specifically in NAFLD patients[67,68]. The original panel was modified by excluding age and naming it enhanced liver fibrosis panel (ELF), and its diagnostic performance was tested in a cohort of 192 patients. The ELF panel had very good performance in distinguishing severe fibrosis (Stage F3-F4) with an AUROC of 0.90 (95% CI 0.84-0.96), while AUROC for detecting moderate and severe fibrosis together (F2-F4) was 0.82 (95% CI 0.75-0.88). Diagnostic accuracy varied with various cut-off points tested, and if cut-offs with 90% sensitivity and specificity for severe fibrosis detection were selected, 86% of study patients would have avoided a liver biopsy, with 76% correctly classified. The study also suggested that the addition of simple parameters, the ones included in previously mentioned NAFLD fibrosis score, could augment the diagnostic performance of the ELF panel, although additional studies with larger sample size would be required to confirm this.

CONCLUSION

Due to the very high prevalence of the disease and numerous difficulties related to establishing the diagnosis, NAFLD remains undiagnosed or incompletely defined in a large number of cases. Therefore, the search for the means to noninvasive diagnosis of different forms of NAFLD is a matter of uttermost importance. It is gaining even greater significance in the light of recent advances in the treatment of NASH, as the research efforts are finally starting to provide us with definite treatment

options. Recently published study with vitamin E and pioglitazone, as well as other current treatment trials place the necessity of establishing a correct diagnosis and not missing NASH in a whole different perspective[12]. Furthermore, given the proportion of population with fatty liver and the fact that the presence of NASH in a given patient is often not linked with elevation in liver enzymes, the number of patients in need of a screening becomes daunting. After the insight in all of the aforementioned studies, we can see that some have indeed come very close and demonstrated very good diagnostic performance of certain noninvasive tools. However, the gold standard used in almost all of the studies is liver biopsy, and the question that remains is whether we are actually able to accurately assess the performance of noninvasive methods when the gold standard by itself has significant flaws. These flaws were very clearly demonstrated in a study of sampling variability in NAFLD by Ratziu *et al*[11]. One can also pose the question: have we maybe found an excellent noninvasive tool already, but are ignorant of the fact due to our incapability to actually see "the absolute truth"? This question has been addressed in a study by Mehta *et al*[69], who calculated the AUROC for a hypothetic liver histology surrogate marker against the biopsy for a range of possible performances of both tests. The authors found that an ideal marker (99% accuracy) could in the best possible setting (sensitivity and specificity of biopsy 90%, prevalence of significant disease 40%) have an AUROC of not more than 0.90. This may mean that, unless an alternative gold standard is found, we might as well be in pursuit of something that isn't there.

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ORIGINAL ARTICLE

Orotate phosphoribosyl transferase mRNA expression and the response of cholangiocarcinoma to 5-fluorouracil

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Abstract

AIM: To determine whether expression of certain enzymes related to 5-fluorouracil (5-FU) metabolism predicts 5-FU chemosensitivity in cholangiocarcinoma (CCA).

METHODS: The histoculture drug response assay (HDRA) was performed using surgically resected CCA tissues. Tumor cell viability was determined morphologically with hematoxylin and eosin- and terminal deoxynucleotide transferase-mediated dUTP nick-end labeling-stained tissues. The mRNA expression of thymidine phosphorylase (TP), orotate phosphoribosyl transferase (OPRT), thymidylate synthase (TS), and dihydropyrimidine dehydrogenase (DPD) was determined with real-time reverse transcriptase-polymerase chain reaction. The levels of gene expression and the sensitivity to 5-FU were evaluated.

RESULTS: Twenty-three CCA tissues were obtained from patients who had been diagnosed with intrahepatic CCA and who underwent surgical resection at Srinagarind Hospital, Khon Kaen University from 2007 to 2009. HDRA was used to determine the response of these CCA tissues to 5-FU. Based on the dose-response curve, 200 μg/mL 5-FU was selected as the test concentration. The percentage of inhibition index at the median point was selected as the cut-off point to differentiate the responding and non-responding tumors to 5-FU. When the relationship between TP, OPRT, TS and DPD mRNA expression levels and the sensitivity of CCA tissues to 5-FU was examined, only OPRT mRNA expression was significantly correlated with the response to 5-FU. The mean expression level of OPRT was significantly higher in the responder group compared to the non-responder group (0.41 ± 0.25 *vs* 0.22 ± 0.12, $P < 0.05$).

CONCLUSION: OPRT mRNA expression may be a useful predictor of 5-FU chemosensitivity of CCA. Whether OPRT mRNA could be used to predict the success of 5-FU chemotherapy in CCA patients requires confirmation in patients.

INTRODUCTION

Cholangiocarcinoma (CCA), a bile duct epithelial tumor, has poor prognosis owing to the absence of an early diagnostic method and effective treatments. The only curative therapy is surgical resection, but most CCA patients are diagnosed at an unresectable stage. Therefore, chemotherapy is the only practical treatment[1].

5-fluorouracil (5-FU) is one of the most common anticancer agents and is used to treat a variety of solid tumors. 5-FU alone or 5-FU-based regimens are widely used to treat CCA patients. As reviewed by Thongprasert[2], the overall response rate and median survival time following treatment with 5-FU for CCA are 10% and 6.5 mo, respectively. In addition, a combination of 5-FU and leucovorin yields a response rate of 32% and a median survival time of 6 mo[2]. Combination therapy of 5-FU with cisplatin consistently yields response rates of 10%-40%, and median survival times are better than those observed with 5-FU alone[2]. Combinations of 5-FU with taxanes or etoposide, however, have not shown convincing superiority over 5-FU alone for CCA treatment[3,4]. Thus, data obtained from these clinical studies revealed relatively poor response rates of CCA to 5-FU-based regimens.

After entering cells, 5-FU is converted to 5-fluorodeoxyuridine monophosphate (FdUMP) through intermediary molecules by thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT)[5-7]. FdUMP then forms a complex with thymidylate synthase (TS), leading to inhibition of DNA synthesis[5]. In addition, 5-FU can be phosphorylated by OPRT to form 5-fluorouridine monophosphate, and then to 5-fluorouridine triphosphate, which is subsequently incorporated into RNA, resulting in RNA dysfunction[8]. In the degradation pathway, 5-FU is metabolized by dihydropyrimidine dehydrogenase (DPD) to an inactive metabolite, 5-fluorodihydrouracil, and subsequently excreted in the urine[9]. The resistance of several cancer types to 5-FU may be due to alterations in the expression of several genes that are involved in the metabolism and action of this drug[10]. Moreover, the expression and activities of these enzymes have been proposed as markers to predict the response to 5-FU of several cancers such as gastric cancers and metastatic colorectal cancer[11-14].

Because 5-FU is the main chemotherapeutic agent for treatment of CCA, elucidating the molecular mechanism involved in the response to 5-FU may be useful for the treatment of CCA. In the present study, we examined the correlation between mRNA expression of target genes involved in 5-FU metabolism and the chemosensitivity of cancer tissues to 5-FU in 23 CCA patients.

MATERIALS AND METHODS

Chemicals

Hank's balanced salt solution (HBSS), RPMI 1640 medium, fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO BRL (Grand Island, NY, United States). Collagen gel sponges were purchased from Pharmacia and Upjohn (Kalamazoo, MI, United States). 5-FU was provided by Fresenius Kabi Oncology Ltd. (Hayarna, India). The DeadEnd Colorimetric Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) system was purchased from Promega (Madison, WI, United States). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, United States). The TaqMan® gene expression assay kit and TaqMan Universal polymerase chain reaction (PCR) master mix with AmpErase UNG were purchased from Applied Biosystems (Foster City, CA, United States).

CCA tissues

Twenty-three samples of CCA tissues were obtained from patients who had been diagnosed with intrahepatic CCA and who underwent surgical resection at the Department of Surgery, Srinagarind Hospital, Khon Kaen University between 2007 and 2009. The histological types of the CCA tissues were classified according to the World Health Organization classification. Of these patients, 69.6% were men. The median age of the CCA patients was 61 years (range: 42-70 years). Written informed consent was obtained from all patients before the collection of tumor tissues. The study protocol was approved by the Khon Kaen Ethics Committee for Human Research, Khon Kaen University, Thailand (HE500501).

Histoculture drug response assay

After surgery, the tumor tissues were immediately transferred to the laboratory in HBSS containing 100 IU/mL penicillin and 100 μg/mL streptomycin. Histoculture drug response assay (HDRA) was performed as described[15] with some modifications. Cubes of collagen gel sponge (1 cm³) were immersed in 1 mL RPMI 1640 containing 20% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5-FU at final concentrations of 100, 200, and 400 μg/mL in a 24-well plate. After washing six times in HBSS, the tumor tissues were aseptically cut into small pieces using biopsy punches (3 mm diameter), placed on the collagen gel sponges, and cultured at 37 ℃ for 4 d

in a 5% CO_2 atmosphere. Duplicate tissue cultures were performed for each drug concentration. Wells containing culture medium without 5-FU were used as controls.

After 4 d of culture, the viability of tumor cells in the cultured tissues was examined with histology. Hematoxylin and eosin (HE) and TUNEL staining were used to assess cell viability[15]. In brief, tissues were fixed in 4% formaldehyde and embedded in paraffin, and 4-μm tissue sections were cut. Deparaffinized sections were rehydrated, stained with HE and TUNEL, and examined under a microscope. *In situ* TUNEL was carried out according to the manufacturer's instructions. TUNEL-positive cells were quantified in at least four high-power fields (× 40) of randomly selected tissue sections. The total live tumor cells showing anaplastic characteristics with hyperchromatic nuclei/cytoplasm were counted and scored as the percent of the total tumor cells. TUNEL-stained tumor cells were identified as dead cells. The efficacy of 5-FU was calculated and expressed as the inhibition index (% I.I.) using the following formula: % I.I. = (1 - % living tumor cells in 5-FU-treated tumor tissue/% living tumor cells in control tissue) × 100. The % I.I.s at various concentrations of 5-FU ranging from doses of 100-400 μg/mL were determined.

Determination of mRNA expression

The mRNA expression of target genes including those encoding TS, DPD, TP, OPRT and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in CCA tissues was determined by reverse transcription and quantitative real-time PCR. In brief, total RNA of each CCA tissue was isolated using TRIzol reagent according to the manufacturer's protocol. Reverse transcription was performed as described[16].

The mRNA expression of the target gene was determined using the TaqMan® gene expression assay kit according to the manufacturer's instructions. Real-time PCR was performed in 20-μL PCR reactions containing TaqMan Universal PCR master mix, target-specific primers, one TaqMan® MGB FAM™ dye-labeled probe, and 50 ng cDNA. Each PCR was carried out in duplicate. The PCR conditions were 50 ℃ for 2 min and 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 1 min. All data were analyzed using the ABI PRISM 7500 Real-time PCR system sequence detection software v.1.4 (Applied Biosystems). The quantity of target cDNA or GAPDH PCR product was calculated using the corresponding standard curve, and the amount of target cDNA in a given sample was normalized to that of GAPDH cDNA.

Statistical analysis

The Student's *t*-test was used to compare the % I.I. values between the responder and non-responder groups.

RESULTS

Microscopic examination of CCA tissues after HDRA

HE- and TUNEL-stained sections of control and 5-FU treated CCA tissues are shown in Figure 1. After 4 d of culture on the collagen gel sponge, the CCA tissue architecture including cell-to-cell contact was well maintained. Most of the tumor cells were alive and showed anaplastic characteristics with hyperchromatic nuclei/cytoplasm (short arrows). On the other hand, cells with eosinophilic cytoplasm, shrunken (condensed) nuclei (pyknotic nuclei), and fragmented nuclei (karyorrhexis, indicated by long arrows) were found in 5-FU-treated cells. TUNEL-stained tumor cells were identified as dead cells. The mean percentage of living tumor cells in control tissues at day 4 was 83.3% ± 14.2% of those observed at day 0. In addition, the proportion of viable tumor cells in control condition gradually decreased on days 5, 6 and 7 of culture; therefore HDRA of CCA tissues was performed for 4 d.

Response of CCA tissues to 5-FU

The responses of CCA tissues to various concentrations of 5-FU ranging from 100-400 μg/mL were determined using CCA tissues obtained from five patients. Dose-dependent responses of CCA tissues to 5-FU were observed (Figure 2). From these results, a 5-FU concentration of 200 μg/mL was selected as the test concentration. The % I.I. values for 5-FU (200 μg/mL) treatment of 23 CCA tissues are shown in Figure 3. The median % I.I. value was selected as the cut-off to classify CCA tissues as responders or non-responders (Figure 3).

Relationship between the expression of target genes and the sensitivity to 5-FU

For each CCA tissue, mRNA expression was quantified with real-time PCR using specific TaqMan probes for genes encoding enzymes involved in the 5-FU metabolic pathway, including TS, DPD, TP and OPRT. Moderate variability in the expression levels of TS, TP and OPRT mRNA normalized to GAPDH expression among individual samples was observed (24-fold, range: 0.05-1.22; 33-fold, range: 0.13-4.27, and 17-fold, range: 0.06-1.02, respectively), whereas high variability was observed for DPD expression (135-fold, range: 0.05-6.76).

Scattered, overlapping expression levels of these genes were observed in the responder and non-responder groups. However, the mean expression level of OPRT was significantly higher in the responder group compared to the non-responder group (0.41 ± 0.25 *vs* 0.22 ± 0.12, $P < 0.05$, Figure 4). The mean expression levels of TS, DPD, and TP appeared higher in the responder group compared to the non-responder group, but the differences were not statistically significant (0.26 ± 0.32 *vs* 0.18 ± 0.12, $P = 0.43$; 1.73 ± 1.96 *vs* 0.74 ± 0.50, $P = 0.12$; and 1.60 ± 1.16 *vs* 1.02 ± 0.72, $P = 0.16$, respectively; Figure 4).

DISCUSSION

5-FU is phosphorylated in cells to become an active metabolite that inhibits DNA synthesis and induces RNA dysfunction[5,8]. Intra-tumoral gene expression and activities of several enzymes related to 5-FU metabolism correlate with sensitivity to this drug for the treatment of several cancers[11-14]. Of the genes we studied, OPRT seems to have predictive power and may be a promising

Control   5-FU 200 μg/mL

HE
staining

TUNEL
staining
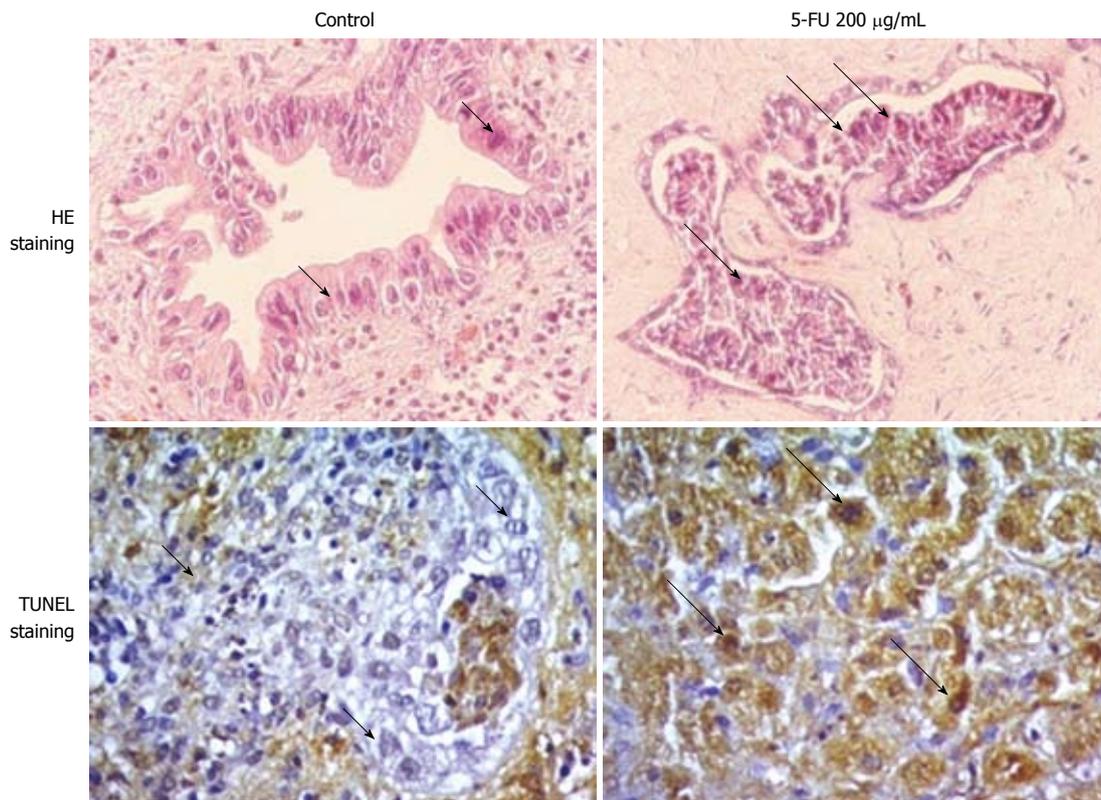
Figure 1 Photomicrographs (40 ×) of hematoxylin and eosin-stained and terminal deoxynucleotide transferase-mediated dUTP nick-end label-stained sections of cholangiocarcinoma tissues cultured on collagen gel sponges for 4 d in the absence or presence of 5-fluorouracil. Tumor cells with anaplastic characteristics showing hyperchromatic nuclei/cytoplasm are indicated with short arrows. Cells with eosinophilic cytoplasm with shrunken (condensed) nuclei (pyknotic nuclei) and fragmented nuclei (karyorrhexis) are indicated with long arrows. 5-FU: 5-fluorouracil; HE: Hematoxylin and eosin; TUNEL: Terminal deoxynucleotide transferase-mediated dUTP nick-end label
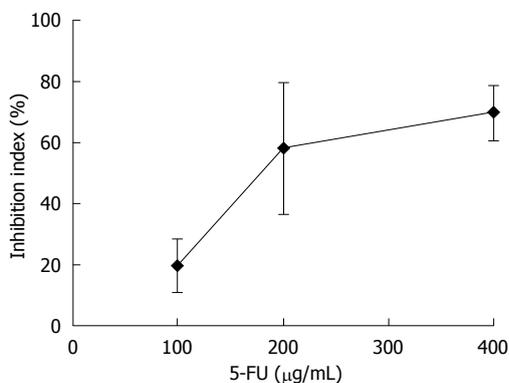
Figure 2 Dose-response-curve of cholangiocarcinoma tissues to 5-fluorouracil. Sensitivities of five cholangiocarcinoma tissues to 5-fluorouracil (5-FU) are shown as the percent of inhibition index (% I.I.), which was evaluated at doses of 100 μg/mL, 200 μg/mL, and 400 μg/mL using histoculture drug response assay. Data are the mean ± SD of two independent experiments.
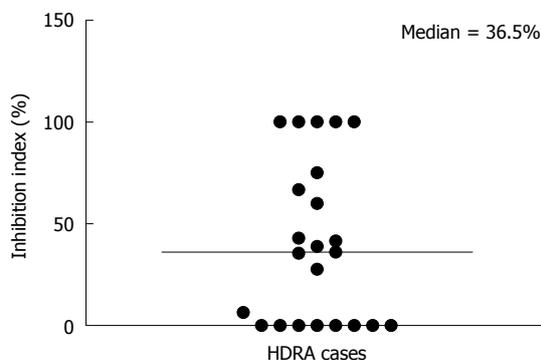
Figure 3 Distribution of percent of inhibition index of 23 cholangiocarcinoma tissues to 200 μg/mL 5-fluorouracil. Sensitivities of 23 cholangiocarcinoma (CCA) tissues to 5-fluorouracil (5-FU) were evaluated at 200 μg/mL 5-FU using histoculture drug response assay (HDRA). The median value of % I.I. (36.5%) was selected as the cut-off for classifying these tissues as responders and non-responders. Each circle represents one CCA sample. The median is shown by the long, thin horizontal line.

marker. High enzymatic activity of OPRT in tumor tissues is associated with high sensitivity of urinary bladder cancer[17] and colorectal cancer[18] to 5-FU. In addition, OPRT mRNA or the ratio of OPRT/DPD mRNA is associated with prolonged survival of metastatic colon cancer patients receiving oral tegafur-uracil and leucovorin[11] and colorectal liver metastasis patients receiving intra-arterial chemotherapy with 5-FU[19]. In addition, combined expression of OPRT and TS in pre-chemotherapeutic fresh-frozen samples obtained from primary tumors may predict the response to S-1, an oral DPD-inhibiting fluoropyrimidine, in metastatic gastric cancer patients[20]. Consistent with the previous studies, we observed here that intratumoral expression of OPRT mRNA in the responder group was significantly higher than expression
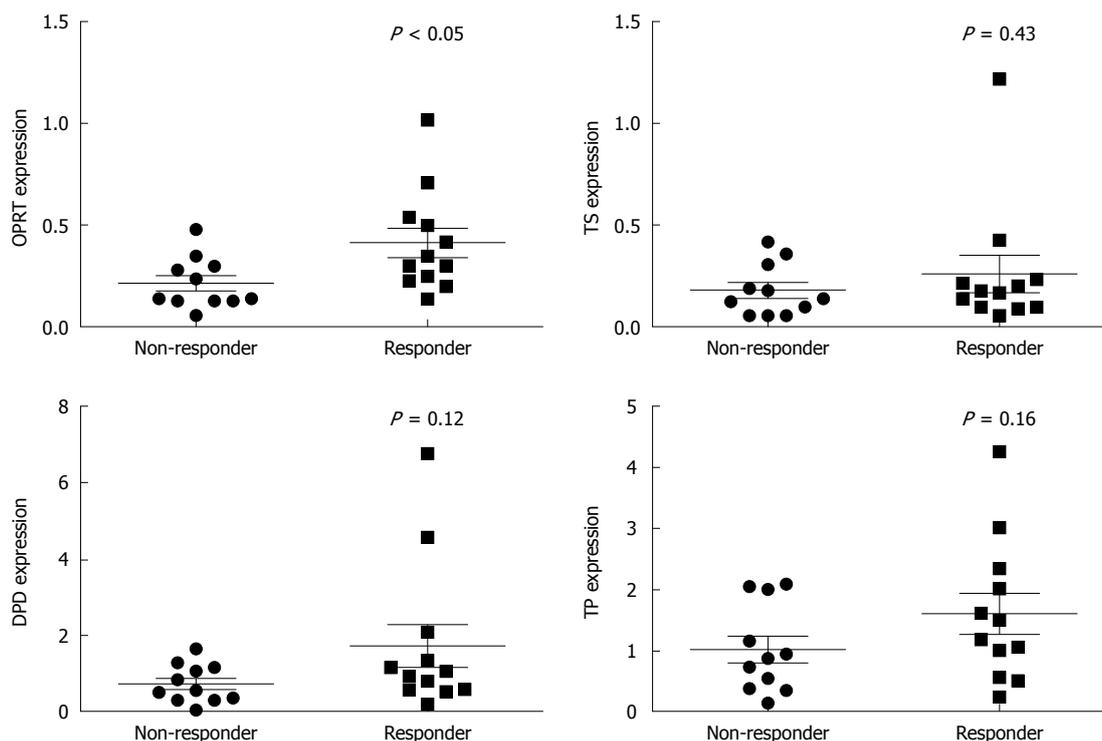
Figure 4 Relationship between chemosensitivity to 5-fluorouracil and mRNA expression of thymidine phosphorylase, orotate phosphoribosyl transferase, thymidylate synthase, and dihydropyrimidine dehydrogenase in 23 cholangiocarcinoma tissues. Chemosensitivity to 5-fluorouracil (5-FU) and mRNA expression were analyzed for 23 cholangiocarcinoma (CCA) tissues using histoculture drug response assay and quantitative real-time reverse transcription-polymerase chain reaction. The responders and non-responders were classified using the median value of the % I.I. as the cut-off point. The mRNA expression of orotate phosphoribosyl transferase (OPRT) was significantly higher in the responder ($P < 0.05$) group. The mRNA levels of thymidylate synthase (TS), and dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) in the non-responder and responder groups, however, were not significantly different. The long, thin lines represent the mean, and the error bars represent the SD values. Each symbol (circles and squares) represents one CCA tissue sample.

in the non-responder group. These results suggest that the OPRT mRNA level in CCA tissues may be a promising predictor of an *in vitro* sensitivity to 5-FU using the HDRA technique. It should be noted that we observed overlapping expression levels of each gene among responder and non-responder tumors.

We observed moderate variability in the expression levels of TS, TP, and OPRT mRNA among individual samples (24-fold, 33-fold and 17-fold, respectively). The moderate differences in OPRT and TS expression levels observed in the present study were similar to those previously observed in gastric carcinoma and colorectal tissues[21,22]. Similar to the variability reported in colorectal[22] and esophageal carcinoma[23], we observed high variability in the expression of DPD among CCA tissues. Genetic polymorphisms of genes encoding TP, DPD, TS and OPRT are well known[24-27]. Variable expression of DPD, TP, TS and OPRT may explained by genetic polymorphisms in these genes.

TP is the first enzyme involved in the metabolic activation pathway of 5-FU to 5-fluorodeoxyuridine (FUdR), which can be phosphorylated by thymidine kinase to form FdUMP[28]. In this study, no relationship was found between the intratumoral expression of TP mRNA and the sensitivity to 5-FU. In addition to TP, that the rate of conversion of 5-FU to FUdR could be influenced by the availability of the TP cofactor, deoxyribose-1-phosphate

(dRib-1-P) in cells[29]. It has been previously demonstrated that the addition of dRib-1-P greatly increases the incorporation of thymidine into DNA and increases the potency of the growth-inhibitory actions of 5-FU[30]. Thus, it may be possible that the amount of dRib-1-P in CCA tissues may be limited. Similar observations have been reported in colorectal tumor that there was no relationship between TP expression and 5-FU sensitivity[31,32].

TS is a key enzyme that catalyzes the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, an important step in DNA synthesis[33]. Colon cancer patients with high TS expression are reportedly un-responsive to 5-FU and show poor prognosis[34]. In contrast, tumoral expression of TS mRNA is associated with response to protracted infusions of 5-FU-based chemotherapy and survival in patients with disseminated colorectal cancer[35]. Consistent with a report on colorectal tumors[36], no relationship between TS expression and sensitivity to 5-FU in CCA *in vitro* was observed in our present study.

DPD is a major enzyme in the metabolism of 5-FU to an inactive metabolite. Some reports have suggested a negative correlation between 5-FU sensitivity and DPD activity in human stomach cancer cells[37]. In patients with advanced colorectal cancer treated with 5-FU/leucovorin, patients who responded to the treatment exhibited low levels and a narrow distribution range of DPD mRNA

expression compared to the non-responder group[32]. In the present study, DPD mRNA expression was apparently higher in the responders than in the non-responders. However, no clear relationship was found between DPD mRNA expression and 5-FU sensitivity.

In conclusion, we developed HDRA as an *in vitro* screening of the response of CCA tissues to 5-FU. We found that the level of OPRT mRNA may be a promising predictor of CCA sensitivity to 5-FU. Whether the OPRT mRNA level could be used as a predictor of the success of 5-FU chemotherapy in CCA patients needs to be confirmed further in patients.

ACKNOWLEDGMENTS

COMMENTS

Background
Cholangiocarcinoma (CCA) has a poor prognosis owing to the absence of effective treatments. Data from clinical studies have revealed that the response rate of CCA to 5-fluorouracil (5-FU) or 5-FU-based regimens is relatively poor. Therefore, a method to identify patients who may benefit from 5-FU is required.

Research frontiers
The histoculture drug response assay (HDRA) was used to determine the response of 23 CCA tissues to 5-FU. By determining the relative expression levels of several genes involved in the action and metabolism of 5-FU, they found that the orotate phosphoribosyl transferase (OPRT) mRNA level was significantly correlated with the response of CCA to 5-FU.

Innovations and breakthroughs
This is the first report to show a relationship between OPRT mRNA expression and the sensitivity of CCA tissues to 5-FU.

Applications
The HDRA may be useful as an *in vitro* test for determining the sensitivity of CCA tissues to anticancer agents. Based on the results from this study, OPRT mRNA expression may be a useful predictor of the chemosensitivity of CCA to 5-FU.

Peer review
This is a good descriptive study in which authors analyze whether expression of certain enzymes related to 5-FU metabolism predicts 5-FU chemosensitivity in CCA. This is an interesting paper of the basic research data of CCA using HDRA.

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ORIGINAL ARTICLE

Increased expression of chondroitin sulphate proteoglycans in rat hepatocellular carcinoma tissues

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Abstract

AIM: To investigate the expression of chondroitin sulphate proteoglycans (CSPGs) in rat liver tissues of hepatocellular carcinoma (HCC).

METHODS: Thirty male Sprague Dawley rats were randomly divided into two groups: control group ($n = 10$) and HCC model group ($n = 20$). Rats in the HCC model groups were intragastrically administrated with 0.2% (w/v) N-diethylnitrosamine (DEN) every 5 d for 16 wk, whereas 0.9% (w/v) normal saline was administered to rats in the control group. After 16 wk from the initiation of experiment, all rats were killed and livers were collected and fixed in 4% (w/v) paraformaldehyde. All tissues were embedded in paraffin and sectioned. Histological staining (hematoxylin and eosin and Toluidine blue) was performed to demonstrate the onset of HCC and the content of sulphated glycosaminoglycan (sGAG). Immunohistochemical staining was performed to investigate the expression of chondroitin sulphate (CS)/dermatan sulphate (DS)-GAG, heparan sulphate (HS)-GAG, keratan sulphate (KS)-GAG in liver tissues. Furthermore, expression and distribution of CSPG family members, including aggrecan, versican, biglycan and decorin in liver tissues, were also immunohistochemically determined.

RESULTS: After 16 wk administration of DEN, malignant nodules were observed on the surface of livers from the HCC model group, and their hepatic lobule structures appeared largely disrupted under microscope. Toluidine blue staining demonstrated that there was an significant increase in sGAG content in HCC tissues when compared with that in the normal liver tissues from the control group [0.37 ± 0.05 integrated optical density per stained area (IOD/area) and 0.21 ± 0.01 IOD/area, $P < 0.05$]. Immunohistochemical studies demonstrated that this increased sGAG in HCC tissues was induced by an elevated expression of CS/DS (0.28 ± 0.02 IOD/area and 0.18 ± 0.02 IOD/area, $P < 0.05$) and HS (0.30 ± 0.03 IOD/area and 0.17 ± 0.02 IOD/area, $P < 0.01$) but not KS GAGs in HCC tissues. Further studies thereby were performed to investigate the expression and distribution of several CSPG components in HCC tissues, including aggrecan, versican, biglycan and decorin. Interestingly, there was a distinct distribution pattern for these CSPG components between HCC tissues and the normal tissues. Positive staining of aggrecan, biglycan and decorin was localized in hepatic membrane and/or pericellular matrix in normal liver tissues; however, their expression was

mainly observed in the cytoplasm, cell membranes in hepatoma cells and/or pericellular matrix within HCC tissues. Semi-quantitative analysis indicated that there was a higher level of expression of aggrecan (0.43 ± 0.01 and 0.35 ± 0.03, $P < 0.05$), biglycan (0.32 ± 0.01 and 0.25 ± 0.01, $P < 0.001$) and decorin (0.29 ± 0.01 and 0.26 ± 0.01, $P < 0.05$) in HCC tissues compared with that in the normal liver tissues. Very weak versican positive staining was observed in hepatocytes near central vein in normal liver tissues; however there was an intensive versican distribution in fibrosis septa between the hepatoma nodules. Semi-quantitative analysis indicated that the positive rate of versican in hepatoma tissues from the HCC model group was much higher than that in the control group (33.61% and 21.28%, $P < 0.05$). There was no positive staining in lumican and keratocan, two major KSPGs, in either normal or HCC liver tissues.

CONCLUSION: CSPGs play important roles in the onset and progression of HCC, and may provide potential therapeutic targets and clinical biomarkers for this prevalent tumor in humans.

INTRODUCTION

Proteoglycans (PGs) are remarkably complex macromolecules consisting of one or more glycosaminoglycan (GAG) chains, which are covalently attached to different core proteins. Depending upon the structures of their GAG side-chains, PGs can be categorized as different groups such as heparan sulphate PGs (HSPGs), chondroitin/dermatan sulphate PGs (CS/DS PGs) and keratan sulphate PGs (KSPGs)[1]. According to their different structures of the core proteins, CS/DS PGs can be further categorized as large aggregating PGs (aggrecan, versican), and small leucine-rich PGs (biglycan and decorin), which have been found to be widely expressed in many tissues including liver.

CS/DS PGs are major components of the cell surface and extracellular matrix (ECM)[1,2]. They perform a myriad of functions ranging from structural roles in the ECM to control of growth factor gradients and the regulation of certain cell processes such as cell adhesion, growth, receptor binding, migration, and interactions with other ECM constituents[3-5]. These, especially the latter two functions, are largely mediated through specific interactions between their charged GAG chains and proteins such as growth factors, cytokines, chemokines, proteinases and their inhibitors[6,7]. In addition, emerging data have revealed that the core proteins of PGs can also form complexes with other proteins, such as integrins and regulate their signaling[7]. Because CS/DS PGs are at the crossroads of many signaling events and the abilities to regulate cell behaviors, they are being extensively investigated for their potential as therapeutic targets for cancers. What has become clear to date is that the functional effects of CS/DS PGs on cancers can range from stimulatory to inhibitory influences[5], depending on the core protein and GAG structures[1,8], the types and stages of cancers and the localizations of the tumors[9].

PGs have been found widespread and abundant in liver tissues[10]. Interestingly, in rats, fetal and early neonatal liver exhibits a completely different PG expression pattern when compared with adult liver tissues, where the synthesis of heparan sulphate (HS) comprises more than 80% and CS less than 5% of total GAGs[11]. In contrast, CS is the major type of GAG synthesized in fetal liver, representing above 50% of total sulfated GAG (sGAG). Moreover, the overall PG production in fetal liver is enhanced two-fold when compared with that in the adult liver tissues. Thus, the synthesis of CS is elevated nearly 30-fold in fetal liver as compared with the adult counterpart[12]. Immediately after birth CS formation decreases rapidly to the adult levels between the 10th and 15th day of postnatal life[13], whereas the production of HS is almost unchanged during perinatal liver development due to a relatively low fractional synthesis of HS GAG in fetal liver[12]. This phenomenon illustrates that CSPG synthesis and expression in liver tissues are cell-type dependent, and the undifferentiated liver cells trend to produce more CSPGs compared with the differentiated hepatocytes in the adult liver tissues.

Interestingly, the expression patterns of PGs including HSPGs and CSPGs are markedly changed under pathological conditions[14]. For example, PGs are abnormally expressed in a wide variety of malignant tumors[9]. In liver, hepatocellular carcinoma (HCC) and hepatic parenchyma adjacent to tumor contain abnormally higher concentrations of CS GAGs than the corresponding healthy tissues, but only with mild alteration in HS expression, indicating that the increase in CS GAGs is a characteristic abnormality in HCC tissues[15-17]. This indicates that the expression of CSPGs plays a pivotal role in the occurrence, progression and metastasis of HCC, and therefore CSPG expression may be a potential marker and treatment target for HCC.

In this study, the expression patterns of different CSPGs including aggrecan, versican, decorin, biglycan in

the liver tissue from a rat HCC model established using N-diethylnitrosamine (DEN) were investigated using histological and immunochemical staining analyses.

MATERIALS AND METHODS

All chemicals were obtained from Xi'an Chemical Reagent Factory (Xi'an, China) unless otherwise stated and were of analytical grade or better.

Animal model preparation

The rat HCC model experimentation was approved by the Animal Ethics Committee, Medical School of Xi'an Jiaotong University. Use of animals in this study was in accordance with the China National Institute of Health publication 85-23 "Guide for Care and Use of Laboratory Animals" (National Research Council, 1996). Thirty male Sprague Dawley rats weighing 248.18 ± 12.32 g (3-4 mo old) were purchased from the Laboratory Animal Center of Medical School, Xi'an Jiaotong University. Rats were acclimated for 7 d before experimentation. Rats were randomly divided into two groups: control group (n = 10) and HCC model group (n = 20). Rats in the HCC model group were intragastrically administrated with 0.2% (w/v) DEN (Sigma, United State) in saline (10 ng DEN per gram body weight) every 5 d for 16 wk, whereas 0.9% (w/v) normal saline was administered to the rats in the control group. All the rats had free access to distilled water. Electrolyte balance between the two groups was maintained through their common dietary food intake.

Sample collection

The weights of the rats were measured every week. After 16 wk from the initiation of the experiment, all the rats were killed under general anesthesia. Hepatic tissues were collected and fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS, 0.16 mol/L NaCl, 0.003 mol/L KCl, 0.008 mol/L Na_2HPO_4, 0.001 mol/L KH_2PO_4, pH 7.3) immediately. The tissues were embedded in paraffin and sectioned at 8 μm thickness.

Histological staining

Sections were deparaffinized and hydrated and either stained with hematoxylin and eosin or Toluidine blue as previously described[18]. After dehydration, sections were mounted using DPX mounting medium (Thermo Fisher Scientific, Loughborough, United Kingdom). Representative regions were photographed under bright field optics using a Leica DMRB light microscope (Leica, Wetzlar, Germany) equipped with digital image acquisition.

Immunohistochemical staining

Immunohistochemical staining was performed using Mouse on Mouse™ Vectastain® Elite® ABC Kits (Vector labs, Peterborough, United Kingdom) according to the manufacturer's protocols. Briefly, sections were incubated with 0.3% (v/v) hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidase activity. After blocking

with mouse immunoglobulin (Ig) blocking reagent for 1 h at room temperature, sections were incubated with rat anti-Versican (Abcam, Cambridge, United Kingdom), mouse anti-Aggrecan, mouse anti-Decorin, mouse anti-Keratocan, mouse anti-Lumican, mouse anti-Biglycan (in house) primary antibodies[19] for 60 min, respectively. For the negative control, the primary antibody was replaced by PBS or 2 μg/mL mouse or rat IgG (DAKO, Ely, United Kingdom). Sections were then incubated with biotinylated goat anti-mouse or rat IgG for 30 min at room temperature. After washing, sections were incubated with Mouse on Mouse™ ABC reagent for 5 min. Sections were then visualized using Vector® NovaRED™ kit (Vector labs, Peterborough, United Kingdom) according to the manufacturer's protocols. Cell nucleuses were counterstained with hemotoxylin. After dehydration, sections were mounted using DPX mountaining medium. Representative regions were then photographed under bright field optics using a Leica DMRB bright field microscope (Leica, Wetzlar, Germany) equipped with digital image acquisition.

Semi-quantitative analysis for versican positive rate in liver tissues

Positive staining rate for versican in liver tissue sections was quantitatively analyzed. Sections of 4 liver tissues from 4 individual rats in each experimental group were taken for analysis. For each liver tissue specimen, three sections were randomly selected, and the positive and negative stained cells in these sections were counted using Image J software (NIH, United States). The percentage of positive cells was then calculated using the equation below: the percentage of positive cells = (positive stained cells)/(positive stained cells + negative stained cells) × 100%.

Semi-quantitative analysis for the intensity of positive staining in tissues

The intensity of positive staining in tissue sections was analyzed by integrated optical density (IOD) using the Image-Pro Plus 5.1 software (Media Cybernetics, United States) as described previously[20] with minor modification. Briefly, four 20 × TIFF-format images from four individual rats in each group were analyzed in a blinded manner. All of the images were taken using the same microscope and camera sets. Image-pro Plus software was used to calculate the average IOD per stained area (μm^2) (IOD/area) for positive staining.

Statistical analysis

Data were presented as mean ± SE, with samples derived from 4 animals in each group. D'Agostino and Pear omnibus normality test was used for normality and equal variances test. Student t test plus Bonferroni's post-test was carried out using GraphPad Prism 4.0 software (GraphPad Software Inc., California, United States). The comparisons of the staining results were performed only between rats from the HCC model and the control groups, but not between the tumor nodules and its adjacent normal liver
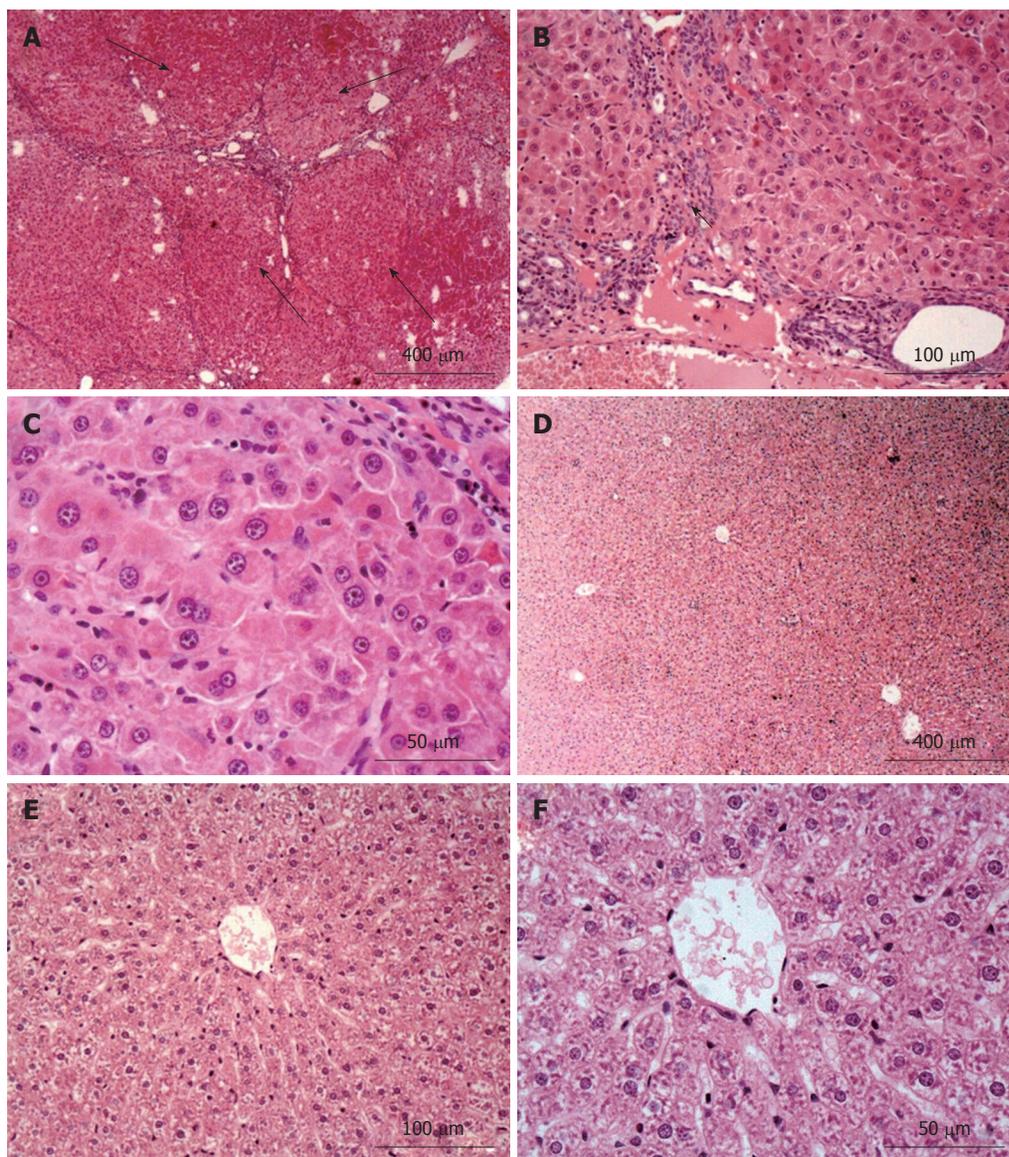
Figure 1 Hematoxylin and eosin staining results for liver tissues from the hepatocellular carcinoma model (A-C) and the control group (D-F). Normal liver structure and cell morphology were observed in the control group. However, apparent hepatoma nodules (long black arrows, A) and fibrosis (short black arrow, B) in the hepatocellular carcinoma model group were observed when compared with that in the control group.

tissues of rats from the same group. Differences were considered significant at $P < 0.05$.

RESULTS

Rat HCC model establishment

After 16 wk administration of DEN, malignant nodules were observed on the surface of the livers in the HCC model group but not in the control group. The average number of macroscopic nodules bigger than 3 mm and 5 mm on the surface of a single liver was 33.4 and 4.9, respectively, with the biggest nodule being approximately 1.5 cm × 1.0 cm × 0.8 cm (Table 1). H and E staining was used to identify and classify the cancerous nodules pathologically according to Edmondson *et al*[21]. As expected, the normal hepatic lobule structure was disrupted and hepatoma nodules (long black arrows) were evident in the tis-

Table 1 Number and size of malignant nodules in rat livers				
Group	**n**	**Nodules ⩾ 3 mm**	**Nodules ⩾ 5 mm**	**The biggest nodule (mm³)**
Control group	10	0	0	0
Model group	14	33.4 ± 7.9	4.9 ± 1.9	122.8

sues from the HCC model group (Figure 1A), which were separated by fibrosis septa (short black arrows, Figure 1B), suggesting fibrosis formation around the tumors. The differentiation of HCC cells was also investigated according to method described by Edmondson. All of the cancer cells in the HCC model group were classified as grade Ⅲ, and there was no hepatic plate-like structure present in the tumor tissues (Figure 1C). In contrast, there was no evidence of macroscopic tumor nodules in the livers from
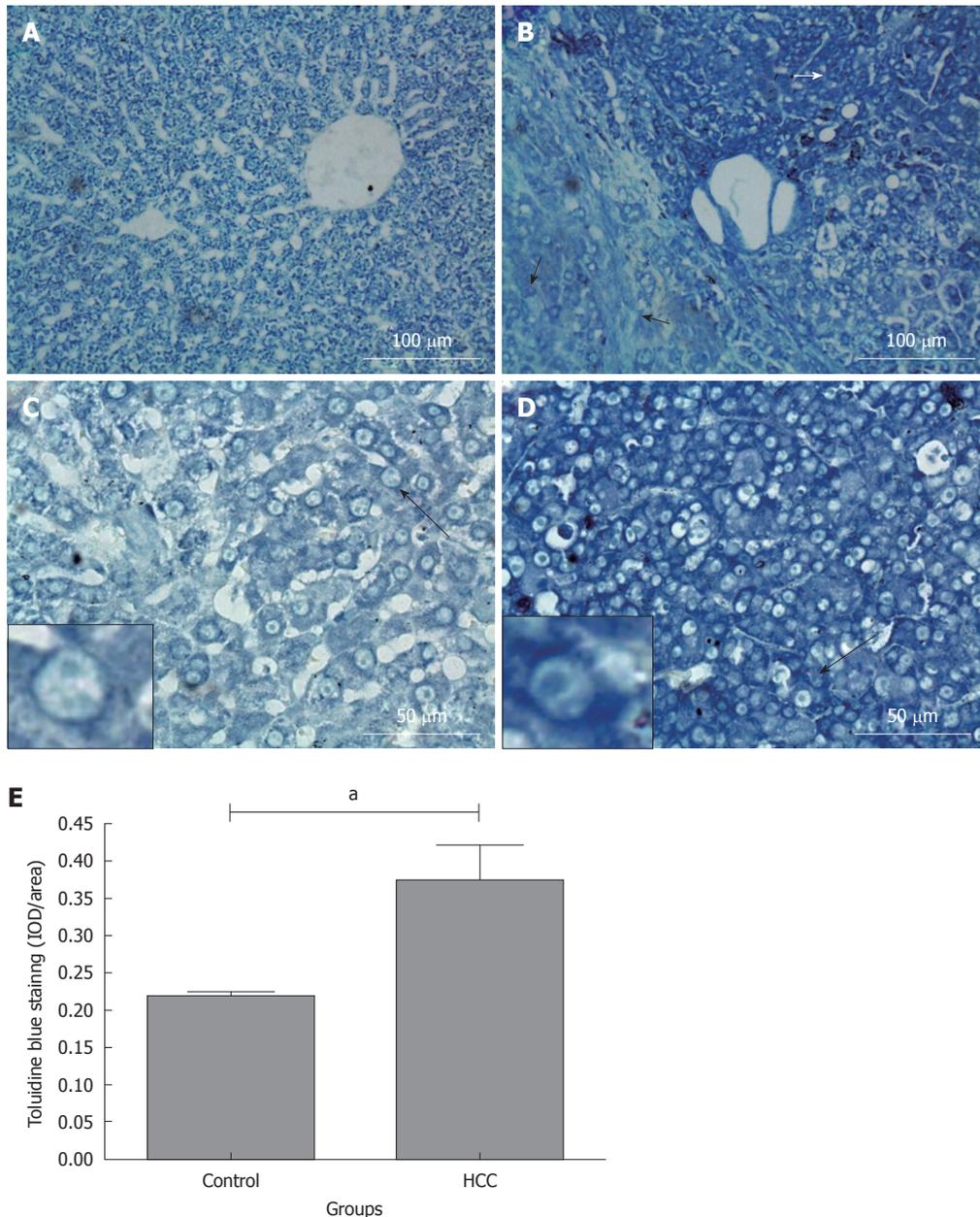
Figure 2 Toluidine blue staining in rat liver tissue sections. Rats were treated with N-diethylnitrosamine for 16 wk to establish a hepatocellular carcinoma (HCC) animal model. Sulphated glycosaminoglycan content in tissues were stained with Toluidine blue. A and C: Control group; B and D: HCC model group. Long black arrows: The cells are magnified in the small boxes. Short white arrow: Hepatoma tissues with intensive Toluidine blue staining; short black arrow: Weaker Toluidine blue staining fibrosis and "relative normal" liver tissues adjacent to the hepatoma nodules; E: Comparison of average integrated optical density (IOD) of toluidine blue staining in liver tissue between control and HCC model group ([a]$P < 0.05$). IOD/area: **Integrated optical density per stained area**.

rats in the control group (Figure 1D), where a normal morphology was observed for hepatic cells, composing a normal liver tissue structure (Figure 1E and F). These results indicated the successful establishment of HCC in rats treated with DEN.

Increased sGAG content in HCC tissues

The contents of sGAG were investigated using Toluidine blue staining, which was evident in liver tissues from both control (Figure 2A and C) and HCC model groups (Figure 2B and D). Positive staining was found in the cytoplasm, cell membrane and/or pericellular matrix

(Figure 2C and D; the cells identified by long black arrows are magnified in the small boxes). Noticeably, Toluidine blue staining in hepatoma tissues (white short arrow, Figure 2B) was stronger than that in the fibrosis and "relative normal liver tissue" (black short arrows, Figure 2B) adjacent to the tumor nodules. Semi-quantitative IOD analysis indicated that there was more Toluidine blue positive staining in the tissues from the HCC model group when compared with the tissues from the control group (0.37 ± 0.05 IOD/area and 0.21 ± 0.01 IOD/area, $P < 0.05$, Figure 2E). This finding demonstrates elevated sGAG content in HCC tissues compared to that in the normal liver tissues. To
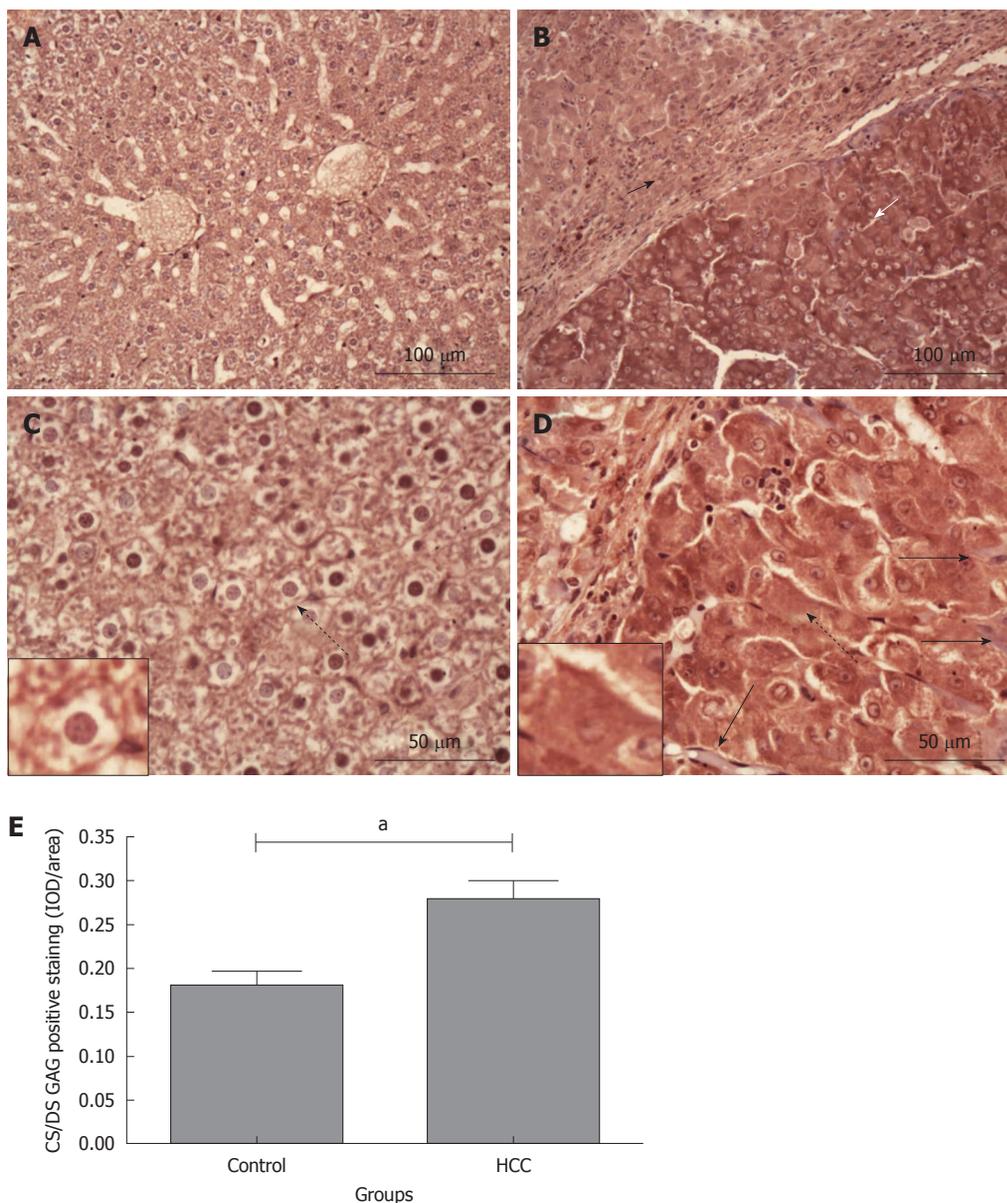
Figure 3 Chondroitin sulphate/dermatan sulphate glycosaminoglycan immunohistochemical staining in rat liver tissues. Chondroitin sulphate (CS)/dermatan sulphate (DS) sulphated glycosaminoglycan (sGAG) content in liver tissues was stained using 2B6 (+) antibody (dark red). A and C: Control group; B and D: Hepatocellular carcinoma (HCC) model group. Long black arrows: Perisinusoidal cells negatively stained by 2B6 antibody; dotted arrows: The cells are magnified in the small boxes; short white arrow: Hepatoma tissues with intensive CS/DS GAG staining; short black arrow: Weaker CS/DS GAG staining in fibrosis and "relative normal" liver tissues adjacent to the hepatoma nodules; E: Comparison of the average integrated optical density (IOD) in CS/DS GAG positive staining in liver tissues between the control and HCC model groups ([a]$P < 0.05$). IOD/area: Integrated optical density per stained area.

further identify the specific expression of sGAG in HCC model tissue, immunohistochemical staining for CS/DS, HS and keratan sulphate (KS) GAG were performed.

Increased CS/DS GAG expression in HCC tissues

The expression of CS/DS GAG chains in normal and HCC tissues was investigated using 2B6 (+) monoclonal antibody[1,19]. As shown in Figure 3, positive staining for 4-sulphated CS/DS GAGs was observed on cell membranes and/or pericellular matrix in the normal liver tissues from the control group (Figure 3A and C; the cell identified with dotted arrow is magnified in the small box). In contrast, 2B6 (+) positive staining was observed in the cytoplasm, cell membrane and/or pericellular ma-

trix (Figure 3B and D; the cell pointed with dotted arrow is magnified in the small box). The expression of 4-sulphated CS/DS GAGs was variable across the HCC tissues, with a stronger staining in the hepatoma nodules (white short arrow, Figure 3B) but a relative weaker staining in its adjacent tissues such as fibrous septa and relatively normal hepatocytes (black short arrow, Figure 3B). Interestingly, these variable distribution patterns in 4-sulphated CS/DS were also observed inside the hepatoma nodules, i.e., several perisinusoidal cells were negatively stained in CS/DS expression (Figure 3D, black long arrows), although most of the hepatoma cells were positively stained. Semi-quantitative IOD analysis for the intensity of positive staining indicated that there was more 4-sulphated CS/DS expres-

sions in the hepatoma nodules when compared with that in the normal tissues from the control group (0.28 ± 0.02 IOD/area and 0.18 ± 0.02 IOD/area, $P < 0.05$, Figure 3E).

Increased HS GAG staining in HCC tissues

We also investigated the expression of HS GAG chains in the tissues from rats in the control and HCC model groups. Similar to the CS/DS GAG staining, HS positive staining was evenly distributed across the normal liver tissue sections from the control group (Figure 4A and D) and mainly localized on the cell membrane and/or pericellular matrix (Figure 4D; the cell identified with a black short arrow is shown at a higher magnification in the small box). However, this "normal tissue" HS distribution pattern was altered and became uneven in the tissues obtained from rats in the HCC model group. In some hepatoma nodules, intensive HS positive staining was observed in the hepatoma cytoplasm, cell membrane, pericellular matrix and even in cell nuclei (Figure 4E; the cell identified with a black short arrow is shown at a higher magnification in the small box). There was no HS positive staining in the fibrous tissue septa (black long arrows; Figure 4B and E). Semi-quantitative IOD analysis indicated that there was a stronger HS staining in these hepatoma nodules than that in the normal liver tissues from the control group (0.30 ± 0.03 and 0.17 ± 0.02, $P < 0.01$, Figure 4G). However in some hepatoma nodules, a relative weaker HS positive staining was observed on the hepatoma cell membrane and/or pericellular matrix (Figure 4C and F; the cell identified with a black short arrow is shown at a higher magnification in the small box), similar to that observed in the normal liver tissues from the control group. In this case, there was no significant difference in the average density of HS positive staining between the HCC model and the control groups ($P = 0.1169$).

KS GAG expression was not altered in HCC tissues

KS is another important sGAG side chains attached to the core proteins of several matrix PGs. In contrast to the CS and HS GAG staining described above, the positive staining of KS GAG chains was weak and there was no difference between control and HCC model groups (data not shown).

Collectively, the results described above demonstrate that there is a significant elevation in the expression of CS/DS and HS but not KS GAG chains in the HCC model tissues when compared with the normal liver tissues. Therefore, we further investigated the expression patterns of different PG core proteins with CS GAGs, including aggrecan, versican, biglycan and decorin.

Increased aggrecan expression in HCC tissues

Aggrecan is a common CSPG found in many musculoskeletal tissues especially in hyaline articular cartilage. Interestingly, aggrecan expression in the liver at the gene level has been reported previously[14,22]. In this study, aggrecan expression in liver tissues was immunohistochemically investigated using a monoclonal antibody [anti-IGD (6B4)]

recognizing the interglobular domain of aggrecan core protein[19,23]. Positive staining for aggrecan was observed in both control and HCC model groups (Figure 5A and B). However, their distribution patterns were different. In the control group where aggrecan positive staining was mainly localized on cell membrane and/or pericellular matrix (Figure 5C; the cell identified with a black short arrow is magnified in the small box). In contrast, there was more intensive aggrecan positive staining in hepatoma cytoplasm, cell membrane and/or pericellular matrix in the tissues from the HCC model group (Figure 5D; the cell identified with a black short arrow is magnified in the small box). Noticeably, there was no or very weak aggrecan positive staining in the fibrous tissue septa between hepatoma nodules (black long arrows, Figure 5B and D). Interestingly, the differences in staining intensity and patterns for aggrecan described above were also observed between the hepatoma tissues and its adjacent "relative normal liver tissues" (Figure 5E; the areas inside the black or red boxes are magnified on the left column). Semi-quantitative IOD analysis indicated that there was more aggrecan positive staining in the tissues from the HCC model group when compared with that in the control group (0.43 ± 0.01 IOD/area and 0.35 ± 0.03 IOD/area, $P < 0.05$, Figure 5F). These results demonstrated that DEN-induced HCC in rat liver increases the aggrecan expression in cells at the protein level, suggesting that there may be a correlation between HCC and aggrecan expression.

Increased versican expression in HCC tissues

Versican is another member of the large aggregating CSPGs family and its expression in rat liver tissues was also investigated by immunohistochemical staining. In contrast to the aggrecan staining, most of the hepatocytes were negatively stained for versican in the liver tissues from the control group (Figure 6A and D). However, a weak versican positive staining was observed on the cell membrane and/or pericellular matrix of some hepatocytes around the central vein (Figure 6D; the cell identified with a black short arrow is magnified in the small box). In the liver tissues from rats in the HCC model group, versican positive staining was observed in some hepatoma cells (Figure 6B and E), and mainly localized in the cytoplasm, cell membrane and/or pericellular matrix (Figure 6E; the cell identified with a black short arrow is magnified in the small box). Statistical analysis indicated that versican positive staining rate in the HCC model group was much higher than that in the control group ($33.61\% \pm 4.90\%$ and $21.28\% \pm 1.79\%$, $P < 0.05$, Figure 6G), although a large number of cells were still negatively stained. Interestingly, the strongest versican positive staining was observed in the pericellular matrix of fibrous tissue septa and portal areas (Figure 6C and F), indicating a different versican distribution pattern between the control and HCC model groups.

Increased biglycan expression in HCC tissues

In normal liver tissues, a moderate positive staining of biglycan was evenly distributed on hepatic membrane
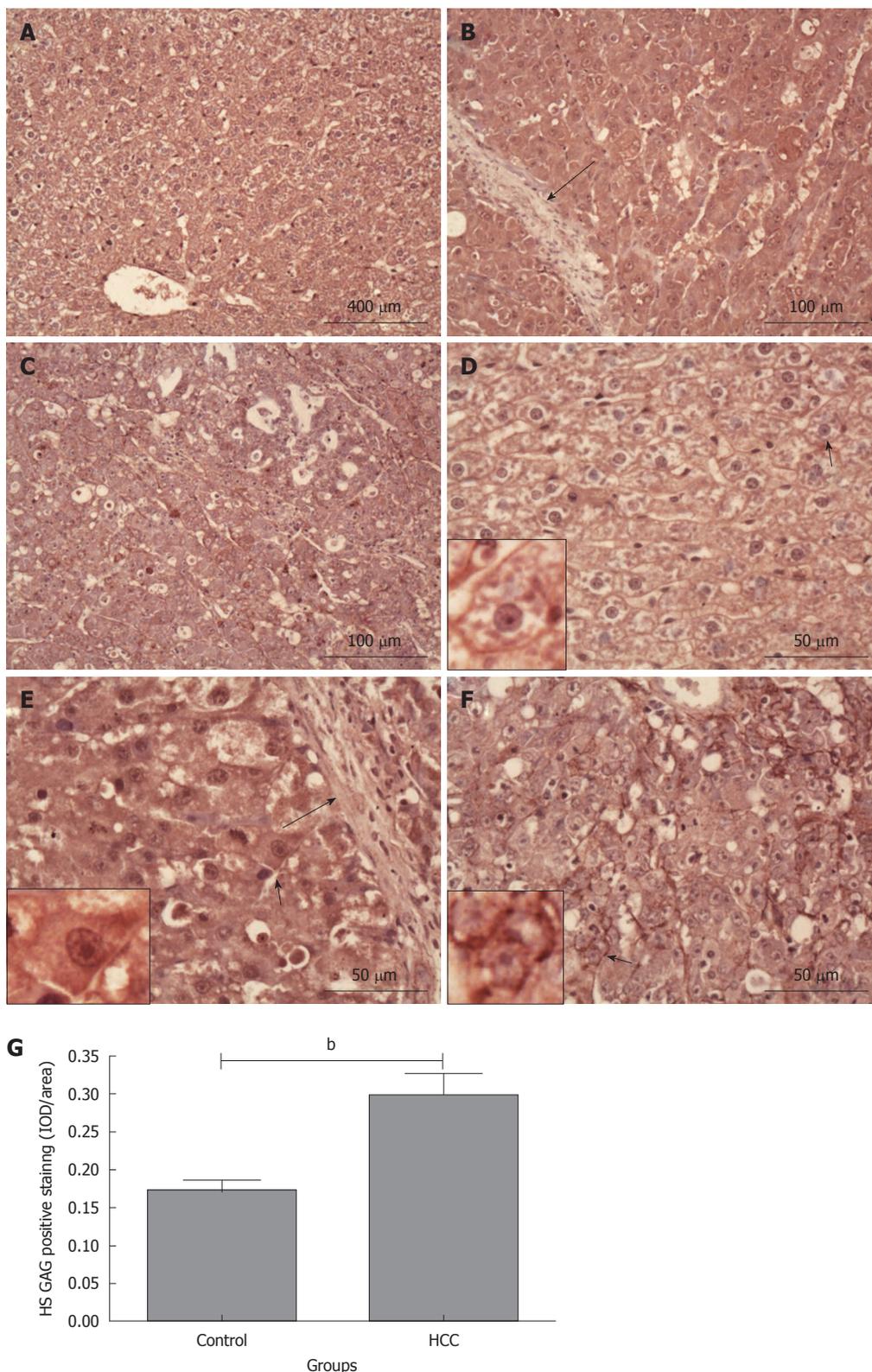
Figure 4 Heparan sulphate glycosaminoglycan staining in rat liver tissues. Heparan sulphate (HS) glycosaminoglycan (GAG) content in tissue was stained using 10E4 antibody (dark red). A and D: Control group; B and E: Hepatocellular carcinoma (HCC) tissues with intensive HS GAG staining; C and F: HCC tissues with relative weaker HS GAG staining. Long black arrows: Fibrous tissue septa; short black arrows: The cells are magnified in the small boxes; G: Comparison of the average integrated optical density (IOD) in HS GAG positive staining in liver tissues between the control and HCC model groups ($^bP < 0.01$). IOD/area: Integrated optical density per stained area.

and/or pericellular matrix in the liver tissues of rats from the control group (Figure 7A). There was limited positive staining in hepatocyte cytoplasm and nuclei (Figure 7C; the cell identified with a black short arrow is magnified in
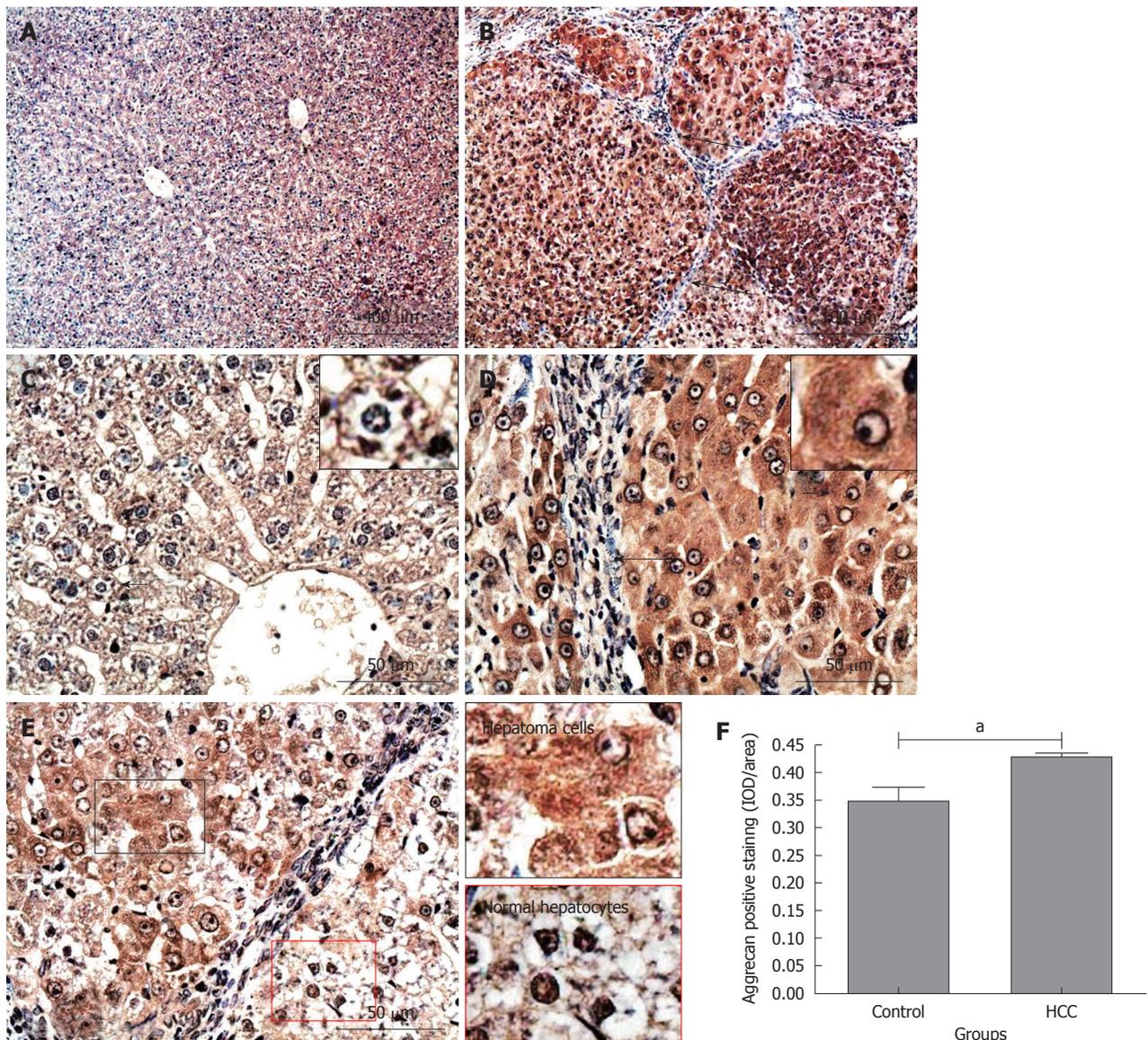
Figure 5 Immunochemical staining for aggrecan in rat liver tissues. Aggrecan positive staining was dark red. A and C: Control group; B, D and E: Hepatocellular carcinoma (HCC) model group. Long black arrows: Fibrous tissue septa; short black arrows: The cells are magnified in the small boxes; E: Areas in black and red boxes are magnified in left column; F: Comparison of the average integrated optical density (IOD) in aggrecan positive staining in liver tissues between the control and HCC model groups ($^aP < 0.05$). IOD/area: Integrated optical density per stained area.

the small box). In contrast, strong biglycan staining was observed in almost all hepatoma cells in tumor nodules from the HCC model group (Figure 7B). The staining was not only on the cell membrane and/or pericellular matrix but also in the cytoplasm (Figure 7D; the cell identified with a white short arrow is magnified in the small box). Interestingly, the differences in staining intensity and patterns for biglycan were also observed between hepatoma cells and its adjacent "relatively normal hepatocytes" (Figure 7E; the areas inside the black or red boxes are magnified on the left column). Semi-quantitative IOD analysis showed that there was significantly more biglycan expression in HCC tissues when compared with that in the normal liver tissues (0.32 ± 0.01 and 0.25 ± 0.01, $P < 0.001$, Figure 7F). There was no intensive biglycan staining in the portal areas and fibrous tissue septa between hepatoma nodules (white long arrow, Figure 7D).

Increased decorin expression in HCC tissues

Similarly to biglycan, decorin positive staining was evenly distributed on hepatic cell membrane and/or pericellular matrix across the whole liver tissue sections from the control group (Figure 8A and C; the cell identified with a black short arrow is magnified in the small box). In the HCC model tissues, intensive decorin positive staining was observed in almost all hepatoma cells (Figure 8B), mainly localized in the cytoplasm, cell membrane and/or pericellular matrix (Figure 8D; the cell identified with a black short arrow is magnified in the small box). This difference described above was also observed between hepatoma cells and its adjacent "relatively normal hepatocytes" (Figure 8E; the areas inside the black or red boxes are magnified on the left column). Semi-quantitative IOD analysis indicated that there was significantly more decorin expression in hepatoma nodules when compared
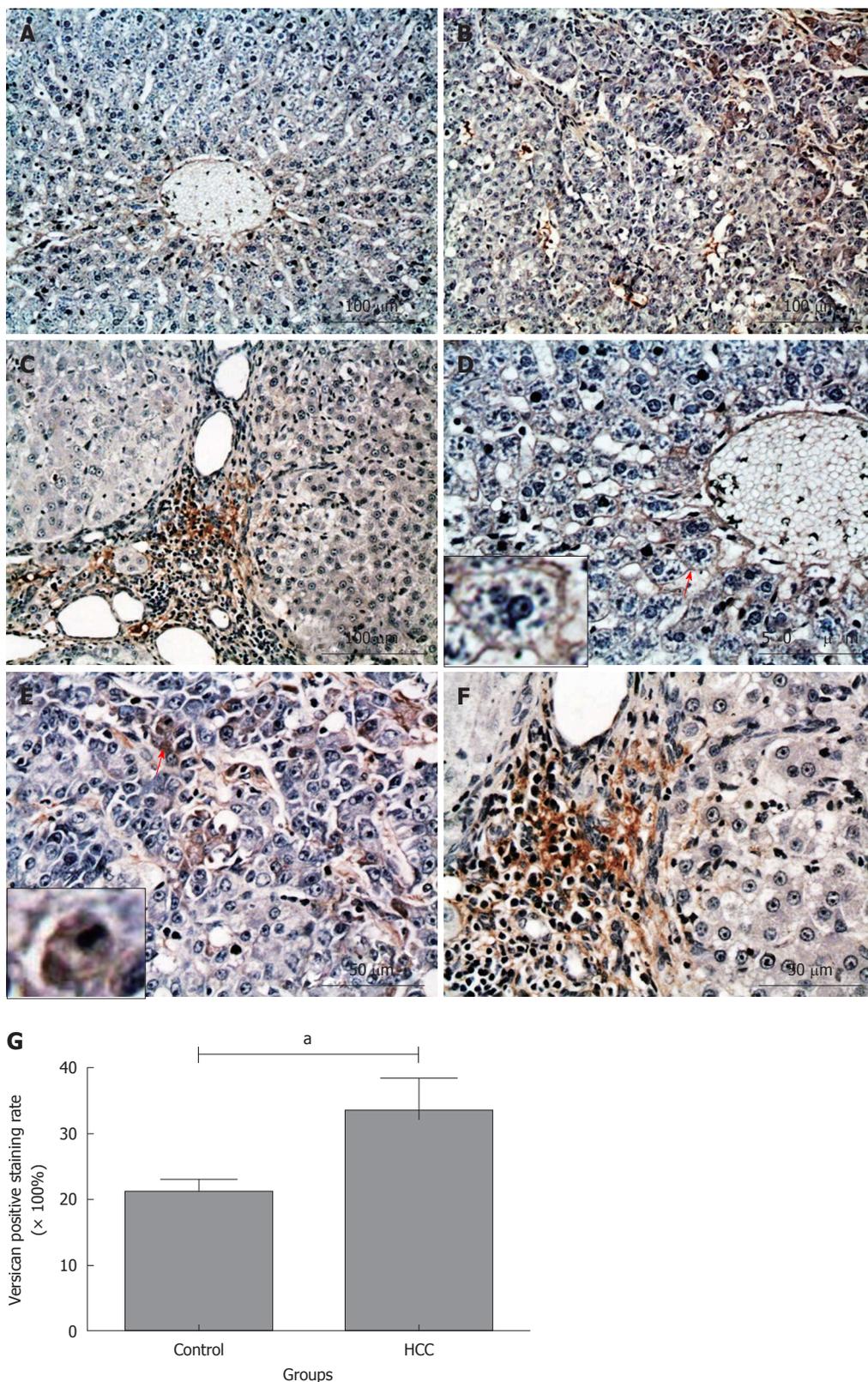
Figure 6 Immunochemical staining for versican in rat liver tissues. Versican positive staining was dark red. A-F: Most of hepatocytes were negatively stained in control group (A and D), whereas more hepatoma cells in hepatocellular carcinoma (HCC) nodules were positively stained (B and E); however, the strongest versican positive staining was observed in the fibrosis septa between hepatoma nodules (C and F). Short red arrows: The cells are magnified in the small boxes; G: Comparison of the positive rate for versican staining in liver tissues between the control and HCC model groups. [a]*P* < 0.05.

with that in the normal liver tissues (0.29 ± 0.01 and 0.26 ± 0.01, $P < 0.05$, Figure 8F). Interestingly, there was no decorin positive staining in the portal areas and fibrous tissue septa between the tumor nodules (Figure 8B and D).
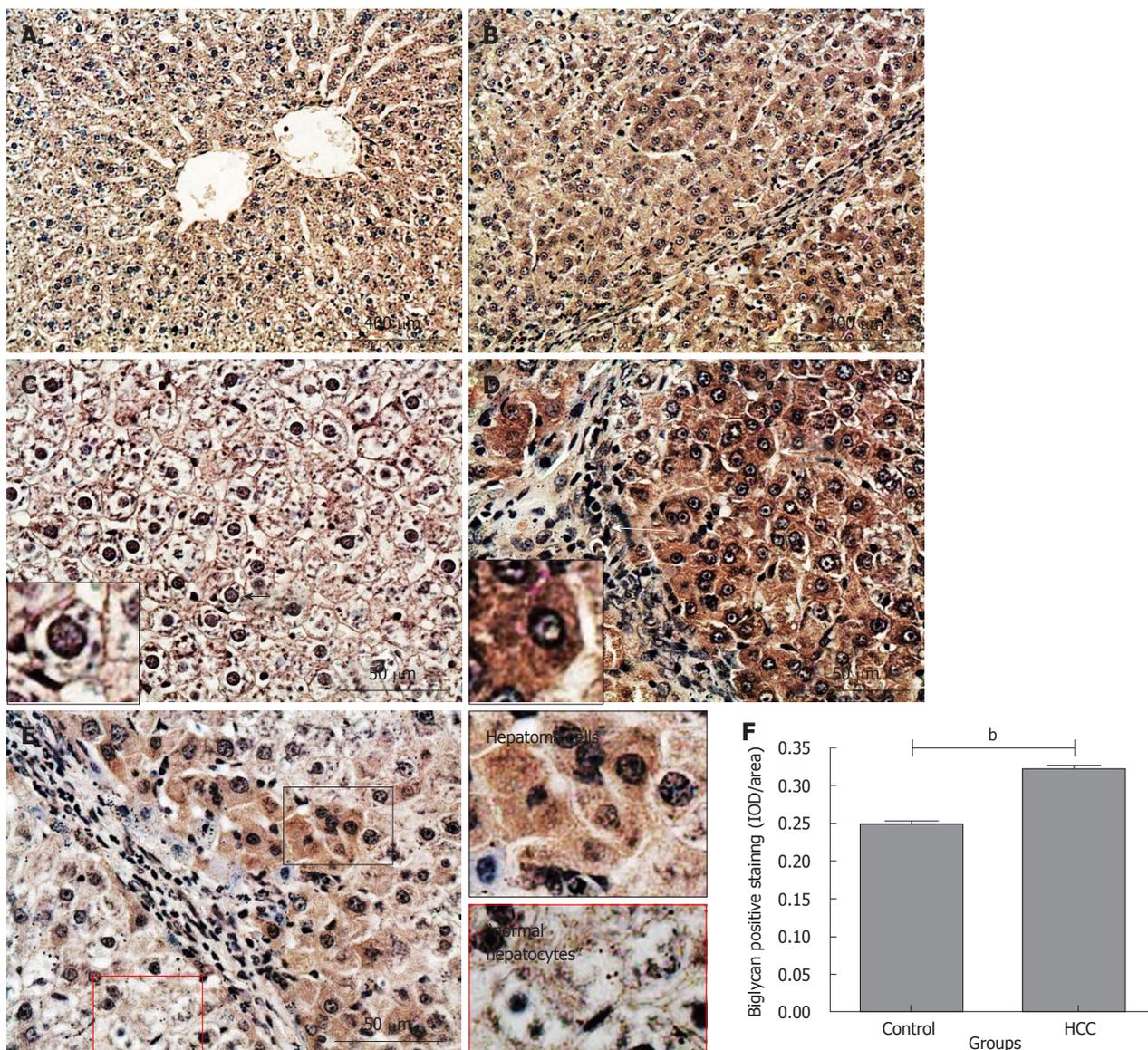
Figure 7 Immunochemical staining for biglycan in rat liver tissues. Biglycan positive staining was dark red. A and C: Control group; B, D and E: Hepatocellular carcinoma (HCC) model group. Long white arrow: Fibrous tissue septa; short black and white arrows: The cells are magnified in the small boxes; E: Areas in black and red boxes are magnified in left column; F: Comparison of the average integrated optical density (IOD) in biglycan positive staining in liver tissues between the control and HCC model groups ([b]$P < 0.001$). IOD/area: Integrated optical density per stained area.

There was no positive staining for keratocan or lumican in rat liver tissues

The expression of keratocan and lumican was also investigated. Consistent with the KS negative staining results, there was no or very weak keratocan or lumican staining in these liver tissues, either from the control or HCC model group (data not shown).

DISCUSSION

The abnormally high expression of CS GAGs in HCC tissues has been known for a long time[16,17] although little is known about the biological mechanisms underlying their increased presence. Interestingly, an accumulation of CS GAG expression has also been observed in other physiological and pathological processes involved in liver development and metabolism. For example, in neonatal liver where premature hepatocytes (hepatic stem cells) still remain as an undifferentiated phenotype, much higher CS GAGs were observed when compared with that in the postnatal liver tissues[13]. Similarly, there is a transient accumulation of CS GAGs during liver regeneration after partial hepatectomy[13] and active fibrosis[24]. All of these examples demonstrate that CSPGs are involved in embryogenesis, regeneration and carcinogenesis of liver. One of the crucial events occurring within these biological processes is the epithelial mesenchymal transition (EMT), a complex molecular and cellular transformation of cell phenotype from differentiated characteristics (mature epithelial cells) to undifferentiated mesenchymal (stem/progenitor cells) features. During this process, cells acquire motility, enhanced migratory capacity/invasiveness[25], and become more stem cell-like[26]. Interestingly, increased production of ECM components such as CS
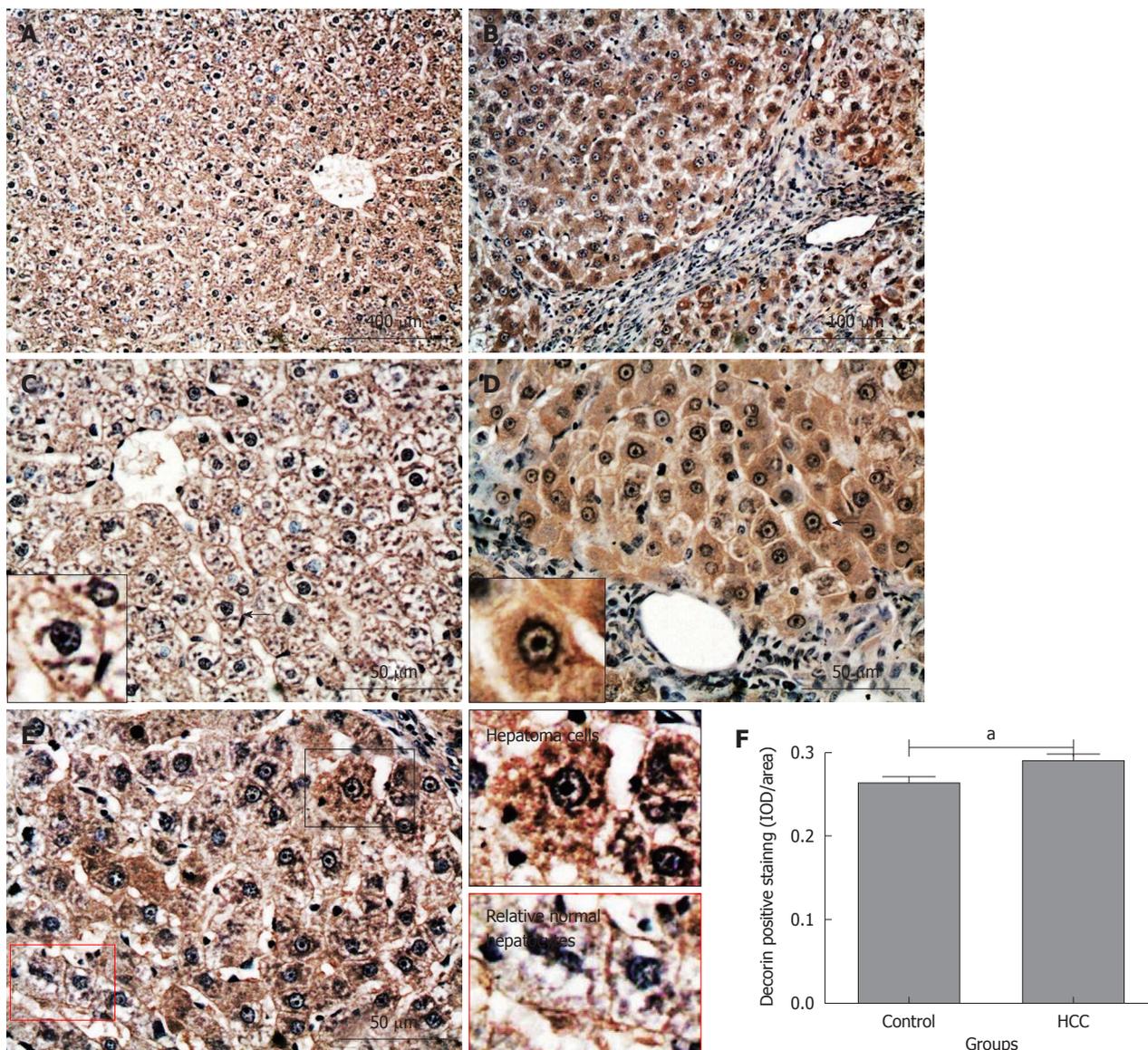
Figure 8 Immunochemical staining for decorin in rat liver tissues. Decorin positive staining was dark red. A and C: Control group; B, D and E: Hepatocellular carcinoma (HCC) model group. Short black arrows: The cells are magnified in the small boxes; E: Areas in black and red boxes are magnified in left column; F: Comparison of the average integrated optical density (IOD) in decorin positive staining in liver tissues between the control and HCC model groups ([a]P < 0.05). IOD/area: Integrated optical density per stained area.

GAGs has been observed during this process[8,27], demonstrating that *de novo* CSPG expression may play a pivotal role during the cell phenotype transformation, notably initiating the development of HCC. However, it is still unclear what the precise CSPG expression patterns are in the hepatoma cells and their relationship with HCC development.

In this study, the Toluidine blue staining results indicated that there was more sGAG content in HCC tissues than that in the normal liver tissues from the control group, which is consistent with previous studies[16,17]. Our further immunostaining with CS/DS, HS and KS antibodies demonstrated that there was a significant increase in CS/DS and HS but not KS GAG expression in HCC tissues. The weak expression of KS GAG in both normal liver and HCC tissues is consistent with the very low staining patterns for keratocan and lumican, two major

KSPGs expressed in the other tissues. A recent study performed on human HCC tissues[28], indicated that KSPGs were not involved in HCC development. Therefore, the increased sGAG content in HCC tissues must be induced by the enhanced production and accumulation of CS/DS and/or HSPGs, which is confirmed by our CS/DS and HS GAG staining. Previous studies have reported the expression of several HSPGs including glypican-3[29], syndecan-4[30] and perlecan[31] are increased in HCC tissues, which coincides with our HS staining results. However, little is known for the expression patterns of specific CSPGs in HCC, therefore our following investigation was mainly focused on CSPG expression.

Aggrecan gene expression has been previously reported in liver tissues[14,22]. Our results demonstrate, for the first time at a protein level, the positive expression of aggrecan in liver tissues, which was mainly localized on

the cell membrane and/or pericellular matrix in normal hepatocytes. However, in the HCC model group there was much stronger aggrecan staining in the cytoplasm, the cell membranes and/or pericellular matrix in HCC hepatoma cells, indicating an elevated aggrecan production and accumulation in HCC tissues. The function of this increased aggrecan presence is not clear but a previous study has suggested that the expression of aggrecan in tumors may be a result of EMT[27]. Moreover, aggrecan production is mediated by different growth factors such as transforming growth factor β in hepatocytes[22,32], which has been identified as a promoter for both HCC-related fibrosis and angiogenesis[32,33]. Interestingly, there was very low aggrecan expression in the fibrous tissue septa between hepatoma nodules, consistent with previous studies showing that the formation of fibrous septa arises from myofibroblasts[34], which have a low expression of CS/DS PGs[35].

In contrast to the increased aggrecan expression, versican content in hepatoma cells was variable with the most intensive staining mainly localized in the ECM of fibrous septa between hepatoma nodules. This finding is novel as little is known about versican expression in HCC tissues. Interestingly, versican expression was observed around the central veins and portal areas, illustrating there may be a close relationship between versican and HCC metastases. This is consistent with previous studies, where an elevated expression of versican was observed in the ECM of other tumor tissues including breast[36] and prostate[37], and correlated with metastases[38]. The mechanism as to how versican promotes metastases is not clear. However, the deposition of versican in the tumor stroma, particularly in the hyaluronic acid rich region, will lead to the structural aggregation of tumor matrix and modulation of cellular attachment and motility, therefore supporting cancer cell growth, proliferation, migration and differentiation, all processes vital for tumor development and metastases[5,36].

Both biglycan and decorin are the members of the small leucine-rich proteoglycan family. They are usually associated with growth factor binding[4] and collagen fibrillogenesis[39,40]. Therefore, it is not surprising that an elevated biglycan and decorin expression was observed during liver fibrosis[10,41,42]. However, our results showed that the major positive staining of decorin and biglycan was localized in the hepatoma cells instead of fibrosis septa between hepatoma nodules, suggesting that the expression of biglycan and decorin may play different roles in HCC occurrence and liver fibrosis. The association between biglycan and HCC has not been previously reported; however, elevated expression of biglycan may correlate with the aggressiveness and poor prognosis of the other cancers[43]. Varied evidence for the changes in decorin expression in HCC tissues has been previously reported. Kovalszky *et al*[17] and Lai *et al*[44] have found that decorin expression was elevated in HCC tissues. In contrast, Miyasaka *et al*[45] showed that there was a decline in decorin gene expression in HCC. The difference may arise from the different stages of HCC, as previ-

ous studies have showed that decorin can be either pro-angiogenic or anti-angiogenic in tumors[46]. The precise contributions of biglycan and decorin metabolism during HCC occurrence and metastases have not yet been elucidated. However, the ability of these proteins to interact with the other matrix components and induce ECM remodeling[47] as well as increasing cell proliferation and migration[48] highlights them as an important PG subsets involved in tumor formation and metastases. Alternatively, the increased biglycan and decorin expression may also be a consequence of EMT of hepatoma cells, because a recent study reported higher biglycan and decorin expression levels during a Ras-induced EMT in MDCK cells[49]. Clearly, further studies for the roles of biglycan and decorin in hepatocarcinogenesis are warranted.

Much less is known about the precise role that CSPGs play in the HCC induction and metastases. CSPGs are ubiquitous components of ECM and cell surface, therefore can predominantly interact with a wide variety of key molecules, such as growth factors, cytokines, chemokines, adhesion molecules, and lipoproteins. These interactions regulate biological processes including signaling, cell differentiation, cell-cell or cell-matrix interactions and morphogenesis[50]. In this study, using histological staining, we found a significant increase in the sGAG content in DEN-induced HCC tissues when compared with the normal rat liver tissues from the control group and this increased sGAG content in tumor tissue was mainly induced by elevated expression in CS/DS and HS but not KS GAGs. We further demonstrated that the expression of several CSPGs including aggrecan, versican, biglycan and decorin was elevated in HCC tissues. To our knowledge, this is the first systematical study demonstrating the elevated CSPGs expression in HCC tissues. The experimental data shown here expands our knowledge of the relationships between CS/DS PGs and HCC, and other liver diseases.

COMMENTS

Background
Proteoglycans (PGs) are macromolecules consisting of one or several polysaccharide chains of the glycosaminoglycan (GAG), which covalently attached to a variety of core proteins. They are widely expressed in cells and extracellular matrix in various tissues including liver. According to the difference in GAG side chains, PGs can be categorized as chondroitin sulphate PG (CSPG) and heparan sulphate PG, *etc*. PGs have been found to play a critical role in different malignant tumor progression. However, the effect of PGs on cancer is variable, which can range from stimulatory to inhibitory, depending on their core proteins and GAG types, the sources and stages of cancers and the tumor localizations.

Research frontiers
Previous studies have shown that the expression of CS GAG was increased in hepatocellular carcinoma (HCC) tissues, and inhibition of CS GAG expression in HCC cell line partially abrogates cell ability of migration *in vitro*. This illustrated that CSPGs may play a pivotal role in the occurrence, progression and metastasis of HCC and thereby they may be used as potential markers and treatment target for HCC. The hotspot in this area is their temporal and spatial expression and the mechanism how they are involved in the onset, development and metastasis of HCC.

Innovations and breakthroughs
The authors investigated the expression pattern of different CSPGs including aggrecan, versican, decorin, biglycan in the liver tissues from a rat HCC model

established using N-diethylnitrosamine (DEN). This is the first systematical study demonstrating the elevated CSPGs expression in HCC tissues.

Applications
The study results suggest that the CSPGs could be potential therapeutic targets and clinical biomarkers for HCC in humans in the future.

Terminology
PG is a kind of macromolecule units consists of a "core protein" with one or more covalently attached GAG chain(s); GAGs are long unbranched polysaccharides consisting of a repeating disaccharide unit; Chondroitin sulfate is a sulfated GAG composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid).

Peer review
This is a good descriptive study in which authors investigate the expression of PGs in rats with DEN-induced HCC. The results are interesting and suggest that CSPGs are potential therapeutic targets and clinical biomarkers for HCC.

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ORIGINAL ARTICLE

Effects of *Lactobacillus plantarum* on gut barrier function in experimental obstructive jaundice

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Abstract

AIM: To investigate the mechanisms of *Lactobacillus plantarum* (*L. plantarum*) action on gut barrier in preoperative and postoperative experimental obstructive jaundice in rats.

METHODS: Forty rats were randomly divided into groups of sham-operation, bile duct ligation (BDL), BDL + *L. plantarum*, BDL + internal biliary drainage (IBD), and BDL + IBD + *L. plantarum*. Ten days after *L. plantarum* administration, blood and ileal samples were collected from the rats for morphological examination, and intestinal barrier function, liver function, intestinal oxidative stress and protein kinase C (PKC) activity measurement. The distribution and expression of the PKC and tight junction (TJ) proteins, such as occludin, zonula occludens-1, claudin-1, claudin-4, junction adhesion molecule-A and F-actin, were examined by confocal laser scanning microscopy, immunohistochemistry, Western blotting, real-time fluorescent quantitative polymerase chain reaction assay.

RESULTS: *L. plantarum* administration substantially restored gut barrier, decreased enterocyte apoptosis, improved intestinal oxidative stress, promoted the activity and expression of protein kinase (BDL *vs* BDL + *L. plantarum*, 0.295 ± 0.007 *vs* 0.349 ± 0.003, *P* < 0.05; BDL + IBD *vs* BDL + IBD + *L. plantarum*, 0.407 ± 0.046 *vs* 0.465 ± 0.135, *P* < 0.05), and particularly enhanced the expression and phosphorylation of TJ proteins in the experimental obstructive jaundice (BDL *vs* BDL + *L. plantarum*, 0.266 ± 0.118 *vs* 0.326 ± 0.009, *P* < 0.05). The protective effect of *L. plantarum* was more prominent after internal biliary drainage (BDL + IBD *vs* BDL + IBD + *L. plantarum*, 0.415 ± 0.105 *vs* 0.494 ± 0.145, *P* < 0.05).

CONCLUSION: *L. plantarum* can decrease intestinal epithelial cell apoptosis, reduce oxidative stress, and prevent TJ disruption in biliary obstruction by activating the PKC pathway.

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INTRODUCTION

Biliary tract surgery in patients with obstructive jaundice is associated with high a morbidity and mortality rate[1]. Evidence accumulated over the past several years indicates that the absence of bile in the gastrointestinal tract promotes bacterial overgrowth and increases intestinal permeability, leading to significant translocation of bacteria and endotoxin following bile duct obstruction[2,3]. The mechanism underlying the increased intestinal permeability in obstructive jaundice has been obscure. However, recent experimental studies have shown that the regional decrease in tight junction (TJ)-associated protein levels in the intestinal epithelium[4], increased apoptosis of enterocytes in intestinal crypts[5], and intestinal oxidative stress[6] are the key factors in the pathogenesis of hepatic and intestinal injury in obstructive jaundice[7].

Probiotic bacteria have been shown to have beneficial effects in the intestinal barrier function. For example, live *Bifidobacterium lactis* has been shown to inhibit toxic effects in epithelial cell culture[8]. *Lactobacillus plantarum* (*L. plantarum*) has been found to inhibit epithelial barrier dysfunction and interleukin-8 secretion induced by tumor necrosis factor-α[9] and prevent cytokine-induced apoptosis in intestinal epithelial cells[10]. *L. plantarum* stabilizes the cellular TJ, thereby preventing enteropathogenic *Escherichia coli*-induced redistribution of integral TJ proteins[11]. Based on the excretion of orally administered ^{14}C, White *et al*[12] demonstrated that enteral administration of the probiotic bacterium *L. plantarum* 299 reduced intestinal hyperpermeability associated with experimental biliary obstruction. However, these authors did not clarify the mechanism for the protective effect of the probiotics on the intestinal barrier in obstructive jaundice. A recent clinical study reported that preoperative oral administration of synbiotics could enhance immune responses, attenuate systemic post-operative inflammatory responses, and improve the intestinal microbial environment after hepatobiliary surgery for obstructive jaundice[13].

TJs, which represent the uppermost basolateral connection between neighboring enterocytes, are important components of the epithelial barrier[14]. TJ assembly and paracellular permeability are regulated by a network of signaling pathways that involves different protein kinase C (PKC) isoforms[15]. A substantial body of experimental data indicates that PKC regulates paracellular permeability of the epithelial barrier[16,17]. PKC regulates the assembly of TJ proteins through phosphorylation of zonula occludens-1 (ZO-1)[15]. Seth *et al*[18] suggested that PKCβI activation may be one of the initial events in the probiotic-mediated protection of TJs. PKCε may play a role in the downstream events of the signaling pathway involved in this process. These data suggest that PKC plays a crucial role in the mediation of intestinal epithelial TJ proteins.

This study aims to investigate the effects of *L. plantarum* on the intestinal mucosal barrier, oxidative stress, epithelial TJ-protein structure and phosphorylation, especially its impact on the expression and activity of PKC.

MATERIALS AND METHODS

Reagents

Rabbit polyclonal anti-occludin, rabbit polyclonal anti-junction adhesion molecule (JAM)-A, rabbit polyclonal anti-claudin-1, mouse monoclonal anti-claudin-4, and rabbit polyclonal anti-phosphoserine antibodies were supplied by Zymed (Invitrogen, Carlsbad, CA, United States). Rabbit polyclonal anti-ZO-1 and rabbit polyclonal anti-PKC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Fluorescein isothiocyanate (FITC)-phalloidin was obtained from Sigma (St. Louis, United States). FITC-conjugated secondary antibodies were supplied by Zymed (Invitrogen). Biotin-labeled goat anti-rabbit immunoglobulin G (IgG) and horseradish peroxidase (HRP)-labeled streptavidin were supplied by DAKO (Glostrup, Denmark). All other reagents of analytical grade were purchased from Sigma (St. Louis, United States).

L. plantarum

The *L. plantarum* (strain CGMCC No. 1258) used in this study was a gift from Dr. Xiao-Ming Hang (Onlly Institute of Biomedicine, Shanghai Jiao Tong University, Shanghai, China). *L. plantarum* cultures were prepared exactly as described previously[11].

Animals

Forty male albino Wistar rats weighing 250-320 g were purchased from Fudan University Medical Animal Center (Shanghai, China). They were housed in stainless-steel cages, three rats per cage, at controlled temperature (23 ℃) and humidity and with a 12 h/12 h dark/light cycle. They were maintained on a standard laboratory diet with tap water ad libitum, except for an overnight fast before surgery. The study was conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and it was approved by the Ethics and Research Committee of Shanghai Sixth People's Hospital, Shanghai, China.

Experimental design

Animals were randomly divided into five groups of eight rats each as described below.

Group Ⅰ, sham-operation: A 2.0-cm upper midline abdominal incision was made, and the common bile duct (CBD) was freed from the surrounding tissues without ligation or transection.

Group Ⅱ, bile duct ligation: The CBD was double ligated in its middle third with a 4-0 silk suture and transected between the two ligatures.

Group Ⅲ, bile duct ligation + *L. plantarum*: After bile duct ligation (BDL), a volume of 10 mL live *L. plantarum* (activity, 2×10^8 CFU/mL) divided into two equal doses was administrated daily to the rats by gavage for 10 d. After 10 d, the animals were sacrificed under ketamine anesthesia.

Group Ⅳ, BDL + IBD: The CBDs of animals were ligated and isolated. A polyethylene tube PE-10 (American Health and Medical Supply International Corp. Co., Ltd., Scarsdale, New York, United States) was inserted into the proximal CBD in a cephalad direction and fixed. The drainage end was tied and positioned in the right hepatorenal recess. After 5 d of obstructive jaundice, the abdomen was reopened through the previous incision. After releasing the biliary obstruction by transecting the tube, a distal 3-cm segment of the catheter was inserted into the duodenum for internal biliary drainage. The animals were sacrificed after another 5 d.

Group Ⅴ, BDL + IBD + *L. plantarum*: Ten days after BDL, live *L. plantarum* was infused as described for Group Ⅲ.

All non-*L. plantarum* control groups, including sham-operation (SHAM), were gavaged with the same volume of the same vehicle (Dulbecco's phosphate buffered saline) used for the *L. plantarum* groups. The animals were sacrificed after 10 d.

All surgical procedures were performed under strict sterile conditions and ketamine anesthesia. At the end of the experiment on day 10, 4-5 mL blood sample was collected from each animal by puncturing the portal vein.

Serum total bilirubin and alanine aminotransferase measurement

The serum total bilirubin and alanine aminotransferase (ALT) levels were determined using a kit (Jiancheng Biological Co., Ltd., Nanjing, China) and a Hitachi Model 7600 series automatic analyzer (Hitachi Co., Tokyo, Japan).

Plasma endotoxin measurement

Endotoxin concentrations were determined using a quantitative chromogenic Limulus Amebocyte Lysate test kit (Shanghai Med. and Chem. Institute, Shanghai, China). Samples were processed according to the manufacturer's instructions[19].

Plasma D-lactate and plasma diamine oxidase measurement

Plasma *D*-lactate levels were measured by an enzymatic spectrophotometric assay[20] using a serum D-lactate quan-

titative colorimetric detection kit according to the manufacturer's instructions (GMS19038.6, Genmed, Boston, MA, United States). Results were expressed as mol/mL. Plasma diamine oxidase (DAO) activities were determined with an enzymatic spectrophotometric assay[21] using a Serum DAO detection kit according to the manufacturer's instructions (Jiancheng Biological Co., Ltd., Nanjing, China). Results were expressed as U/L.

Plasma reduced glutathione/oxidized glutathione measurement

Plasma glutathione (GSH) and glutathione (GSSG) were determined by an enzymatic spectrophotometric assay[22] using the GSH and GSSG detection kits according to the manufacturer's instructions (Jiancheng Biological Co., Ltd.). Results were expressed as mol/mL.

Detection of superoxide dismutase and malondialdehyde in the ileum

Superoxide dismutase (SOD) activity was detected using Sun *et al*'s[23] nitroblue tetrazolium method. Malondialdehyde (MDA) levels were measured using the thiobarbituric acid test according to Ohkawa *et al*[24]. Intestinal tissue samples were thawed, weighed, and homogenized 1:9 (w/v) in 0.9% saline. The homogenates were centrifuged at $3000 \times g$ for 10 min at 4 ℃, and the supernatant was removed for the measurement of MDA content, SOD activity, and total protein. Total intestinal protein concentration was determined by a Coomassie blue method, with bovine serum albumin (BSA) as a standard. SOD activity and MDA levels were detected with kits according to the manufacturer's instructions (Jiancheng Bioengineering Ltd., Nanjing, China). Results were expressed as U/mg protein and nmol/mg protein.

Light microscopy

Samples 1 cm in length were collected from the terminal ileum. To avoid mucosal damage, the intestinal lumen was carefully cannulated and gently washed with normal saline before sampling. Specimens were fixed by immersion in 10% buffered formaldehyde solution and embedded in paraffin. Sections (5 μm thick) were cut and stained for routine light microscopy using HE.

Transmission electron microscopy

Samples 3-4 mm in length were collected from the terminal ileum. These samples were longitudinally cut and immersed in 2.5% phosphate-buffered glutaraldehyde solution for 24 h at 4 ℃. Specimens were then washed with phosphate-buffered solution (PBS), fixed in 1% osmium tetroxide for 2 h at 4 ℃, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 for 48 h. Sections (1 μm thick) were cut and stained with methylene blue. Ultrathin sections were then cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed under transmission electron microscopy (TEM).

Terminal deoxyuridine nick-end labeling assay

Four-μm thick sections were collected on poly-L-lysine-

coated glass slides. The nuclear DNA fragmentation of apoptotic cells was labeled *in situ* by the terminal deoxyuridine nick-end labeling immunohistochemical method[25] using an ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (CHEMICON, Billerica, MA, United States) according to the manufacturer's instructions.

Immunofluorescence microscopy

Terminal ileum tissues were fixed in 3% paraformaldehyde for 3 h, washed with PBS, and embedded in paraffin. Sections (5 μm thick) were cut and attached to glass slides. After deparaffinization and rehydration, sections were permeabilized with 0.2% Triton X-100 in PBS for 20 min. Slides were washed with PBS extensively and blocked with 5% normal goat serum PBS containing 0.05% Tween-20 and 0.1% BSA for 20 min at room temperature. Primary antibodies were added to the slides and incubated overnight at 4 ℃ in a humidity chamber. After washing, sections were incubated with FITC-conjugated specific secondary antibody (Sigma) at room temperature for 2 h in the dark. The slides were again washed extensively and then mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, United States). Sections were observed under a confocal laser microscope (LSM 510, Zeiss, Jena, Germany).

Expression of PKC by immunocytochemical staining using labelled streptavidin biotin method

After the rats were sacrificed, terminal ileum tissues were excised and fixed in Bouin's solution and embedded in paraffin. Immunohistochemistry was performed on 5-μm thick paraffin sections. After deparaffinization and dehydration, endogenous peroxidase was blocked with 30 mL/L H$_2$O$_2$ for 15 min. After blocking of nonspecific binding sites with 5% normal goat serum, slides were incubated with specific primary antibody overnight at 4 ℃. Primary antibodies were diluted 1:50 (rabbit polyclonal anti-human PKC, Santa Cruz Biotechnology, Inc., United States) in PBS. Next, the slides were washed three times for 5 min each with PBS and incubated with biotinylated goat anti-rabbit IgG at 37 ℃ for 30 min, washed as before, and developed with HRP-labeled streptavidin. The incubation and the subsequent washing were exactly the same as done before. Finally, diaminobenzidine chromogen, a peroxidase substrate, was added for color development. The reaction was stopped with a tap water rinse. The sections were counterstained with hematoxylin and mounted for examination.

Western blotting analysis

Terminal ileum samples were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer [150 mmol NaCl, 50 mmol Tris·HCl (pH 7.4), 0.5 mmol phenylmethylsulfonyl fluoride, 2.4 mmol EDTA, and 1 mmol sodium orthovanadate with 1% nonidet-40 (NP-40) and Sigma protease inhibitor cocktail (1:100)] for 30 min at 4 ℃. After centrifugation at 10 000 × *g* for 10 min at 4 ℃, the protein concentration of each sample was quantified by the Bradford method. An equal amount of total protein was separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to a nitrocellulose membrane. After blocked overnight in tris-buffered saline (TBS) containing 0.05% tween (TBS-T) and 5% dry powdered milk, membranes were washed three times for 5 min each with TBS-T and incubated for 2 h at room temperature in primary antibody (rabbit anti-claudin-1, rabbit anti-occludin, rabbit anti-JAM-A, or rabbit anti-ZO-1). After three washes with TBS-T, the membranes were incubated for 1 h with HRP-conjugated secondary antibody. Following two washes with TBS-T and one wash with TBS, the membranes were prepared for visualization of protein by the addition of enhanced chemiluminescence (ECL) reagent (Amersham, Princeton, NJ, United States). Densitometric analysis was performed using an Alpha Imager 1220 system (Alphainotech Co., San Leandro, CA, United States).

Real-time reverse transcription-PCR

The levels of occludin, ZO-1, claudin-1, claudin-4, JAM-A and UGT1A mRNA were measured by real-time reverse transcription-PCR (RT-PCR) using SYBR1 green[26]. Total RNA was isolated from terminal ileum samples with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Real-time RT-PCR was performed with an ABI prism 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The primers were designed using the Primer Express® Program (Applied Biosystems). Their sequences are shown in Table 1. The following procedure used 2 μg of RNA. In a sterile RNase-free microcentrifuge tube, 1 μL of 20 μm oligo (dT) 15 primer was added to a total volume of 15 μL water. The tubes were heated to 70 ℃ for 5 min, cooled immediately on ice, and spun briefly. The following reagents were added to the annealed primer/template: 5 μL of 5 × M-MLV reaction buffer, 1.25 μL of 10 mmol dNTPs and 25 units of RNasin RNase inhibitor, and 200 units of M-MLVRT RNAse H were added to the reagent to yield a 25 μL total reaction volume. All were mixed gently and then incubated for 60 min at 42 ℃ before the reaction was terminated at -20 ℃.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was used as a house-keeping gene control. Separate PCR reactions (25 μL) were conducted for each transcript and contained 2.0 μL cDNA, 12.5 μL of 2 × SYBR Premix Ex Taq™ (TaKaRa, Ltd., Shiga, Japan), and 0.5 μL each of 10 μmol/L gene-specific forward and reverse primers. PCR conditions were optimized to 95 ℃ (30 s), followed by 40 cycles (45 s each) at 95 ℃, 60 ℃ (5 s), and 72 ℃ (30 s), and the reaction was completed at 37 ℃ for 30 s. Five serial dilutions of cDNA were analyzed for each target gene and used to construct linear standard curves. To compensate for variations in the RNA input and in the efficiency of the real-time RT-PCR, we used a normalization strategy based on the house-keeping gene *GAPDH*.

Immunoprecipitation and immunoblotting assays

The terminal ileum tissues were homogenized and ex-

Table 1 Sequences of oligonucleotide primers and conditions for real-time polymerase chain reaction

Gene target	Genbank number (mRNA)	Oligonucleotide[1] (5'- to 3'-)	Annealing temperature (℃)	Product size (bp)
Occludin	NM-031329	F: GCTCAGGGAATATCCACCTATC	60	344
		R: TTCTCCAGCAACCAGCATC		
ZO-1	NM-001106266	F: CCACAGACATCCAACCAGC	60	247
		R: AGCCCAAAGAACAGAAGACC		
Claudin-1	NM-031699	F: GCCTCCAATGCCGTTCT	60	317
		R: TGCCTGCGTCCCTCTTG		
Claudin-4	NM-001012022	F: GTTCCCGCCAGCAACTATG	60	282
		R: CCTTCAGCCCCGTATCCA		
JAM-A	NM-053796	F: CCTCCATCCAAGCCGACA	60	211
		R: CAAAGACCAAATCCCCTGAC		
prkC	NM-001105713	F: GATGGACGGGGTCACGA	60	165
		R: CGCTTGGCAGGGTGTTT		
β-actin	NM-031144	F: CAGGTCATCACTATCGGCAAT	60	144
		R: GAGGTCTTTACGGATGTCAAC		

[1]Primers were designed based on sequences of rat-corresponding genes from the GenBank database. *JAM-A*: Junction adhesion molecule-A; *ZO-1*: Zonula occludens-1.

tracted with the buffer used for Western blotting assays for 30 min at 4 ℃. After centrifugation at 10 000 × *g* for 10 min at 4 ℃, the protein concentration of each sample was quantified by the Bradford method. The supernatant was treated with protein G plus protein A agarose beads (Sigma) and incubated overnight at 4 ℃ with rabbit anti-occludin antibody (Zymed) and protein G + protein A agarose beads. The beads were washed with PBS and ice-cold RIPA buffer, and immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes (Invitrogen). The membranes were blocked with 1% BSA in PBS overnight at 4 ℃ and then incubated with rabbit anti-phosphoserine antibodies (Zymed) for 2 h at room temperature, followed by HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., United States). The reaction was visualized by an enzyme chemiluminescence kit from Pierce (Rockford, IL, United States). Western blotting was performed with an anti-occludin rabbit polyclonal antibody (Zymed) followed by an anti-rabbit secondary antibody coupled with peroxidase (Santa Cruz Biotechnology, Inc.) and ECL. For Western blotting of ZO-1, the same protocol was used with the rabbit polyclonal anti-ZO-1 antibody and a rabbit anti-β-actin antibody (both from Santa Cruz Biotechnology, Inc.).

PKC activity assay
The PKC activity assay was conducted according to the instructions of the PepTag non-radioactive PKC assay kit (Promega, Madison, WI, United States). Briefly, terminal ileum tissues were homogenized and lysed in cold lysis buffer, containing 20 mmol/L tris-HCl, 0.5 mmol/L ethylene glycol tetraacetic acid, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L phenylmethanesulfonyl fluoride, and 10 mg/L leupeptin (pH 7.5). Assays were then performed at 30 ℃ in a total volume of 25 μL containing 5 μL PKC reaction 5 × buffer, 5 μL PLSRTLSVAAK peptide, 5 μL PKC activator, 1 μL peptide protection solution, and 9 μL sample. Reactions were initiated by the addition of the 9 μL sample and terminated after 30 min

by incubation of the reaction mixture at 95 ℃ for 10 min. After added with 1 μL of 80% glycerol, each sample was separated by 0.8% agarose gel electrophoresis at 100 V for 15 min. The intensity of fluorescence of phosphorylated peptides reflected the activity of PKC. All experiments were carried out in triplicate, with each data point representing the results from a separate culture.

Image analysis
Quantification of the immunohistochemical and immunofluorescence staining was performed on stored images of completely scanned tissue sections. Images were acquired with an AxioCam MRc (Carl Zeiss, Jena, Germany) connected to an Axioplan 2 fluorescence microscope (Carl Zeiss), at × 40 magnification. Each microscopic field was individually autofocused before acquisition. Five fields were selected from each slide and a total of five slides per group were examined. All image acquisition and processing were done using custom-written macros in KS400 image analysis software (version 3.0, Carl Zeiss).

Statistical analysis
Results were presented as mean ± SD of three experiments. The data were analyzed using GraphPad PRISM (GraphPad Software Inc., San Diego, CA, United States) and SPSS 11.0 (SPSS Inc., Chicago, IL, United States). All data were analyzed using one-way analysis of variance with Bonferroni/Dunnett T3 *post-hoc* test for multiple comparisons to determine differences between two experimental groups. *P* values < 0.05 were considered to be significant.

RESULTS

General observations
All animals survived throughout the experiment. Bile duct ligated rats were clinically jaundiced within 3 d. At reoperation on day 3, the ligation and division of the CBD were successful in all cases and resulted in significant dilatation of the CBD remnant proximal to the liga-
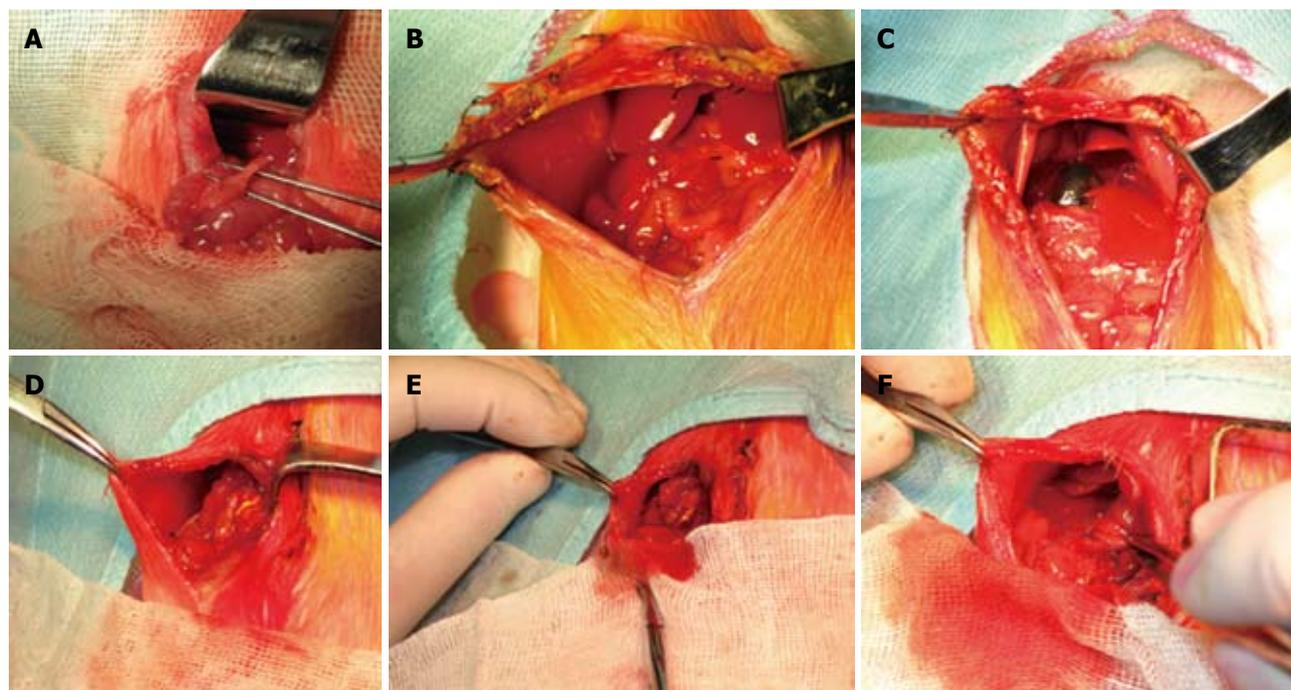
Figure 1 Images of experimental obstructive jaundice and internal biliary drainage. A: Dissection revealing the common bile duct; B: Reoperation after 3 d of common bile duct ligation. Light yellow abdominal ascites were present in the right side of the abdominal cavity; C: The proximal bile duct showed a remarkable expansion (dark blue color) after 3 d of common bile duct ligation; D: The PE-10 polyethylene tube was positioned with the end tied in the right hepatorenal recess; E: Brown bile flowed out while the catheter end was open; F: The distal 3 cm segment of the catheter was inserted into the duodenum for internal biliary drainage.

Table 2 Levels of endotoxin, total bilirubin, alanine transaminase and aspartate transaminase (mean ± SD)

	Group I	Group II	Group III	Group IV	Group V
Endotoxin (ng/mL)	0.58 ± 0.09	17.12 ± 1.09[a]	14.25 ± 0.68	3.05 ± 0.78[c]	1.91 ± 0.54
TBIL (μmol /L)	3.0 ± 1.63	153.83 ± 25.73[a]	132.0 ± 23.09	23.75 ± 5.42[c]	9.0 ± 1.87
ALT (U/L)	60.8 ± 5.69	543.83 ± 184.09[a]	218.38 ± 91.09	118.63 ± 19.72[c]	97.0 ± 10.37
AST (U/L)	130.9 ± 27.42	980.5 ± 663.25[a]	512.75 ± 156.76	437.88 ± 42.41[c]	271.0 ± 28.93

[a]$P < 0.05$ *vs* Group III; [c]$P < 0.05$ *vs* Group V. Group I: Sham-operation; Group II: Bile duct ligation (BDL); Group III: BDL + *L. plantarum*; Group IV: BDL + internal biliary drainage (IBD); Group V: BDL + IBD + *L. plantarum*. TBIL: Total bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase.

ture with obvious cholestatic appearance of the liver. At reoperation on day 10, the CBD diameter had returned to a normal size in the BDL + IBD group, and the cholestatic livers also appeared improved visually (Figure 1).

Serum total bilirubin and ALT levels
Obstructive jaundice led to significantly elevated serum levels of total bilirubin [153.73 mmol/L *vs* 3.0 ± 1.63 mmol/L] and ALT [543.83 U/L *vs* 60.8 ± 5.69 U/L, $P < 0.05$]. Administration of *L. plantarum* significantly reduced levels of total bilirubin (132 ± 23.9 mmol/L *vs* 9.0 ± 1.87 mmol/L) and ALT (218.38 ± 91.09 U/L *vs* 97 ± 10.37 U/L) in the portal serum (Table 2).

Portal endotoxin concentrations
Group II (BDL + *L. plantarum*) animals presented with significantly elevated endotoxin concentrations in portal blood compared with those in Group I (SHAM) ($P < 0.05$). Treatment with *L. plantarum* in the BDL + *L. plantarum* and BDL + IBD + *L. plantarum* groups significantly

reduced endotoxin levels in the portal serum (Table 2).

Plasma D-lactate and plasma DAO measurement
Plasma *D*-lactate levels increased significantly in the BDL group compared with the SHAM group. Administration of *L. plantarum* significantly decreased the plasma D-lactate levels in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Table 3).

DAO activity in the BDL group was significantly higher than that in the SHAM group. Plasma DAO activity became significantly lower after the use of probiotics in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Table 3).

Glutathione redox state
Plasma GSH was significantly reduced in Group II (BDL) animals compared with those in Group I ($P < 0.05$). Administration of *L. plantarum* significantly increased the levels of GSH in the Group IV (BDL + IBD) animals, whereas GSSG was found to be significantly increased in BDL animals. Administration of *L. plantarum* significantly

Table 3 Levels of *D*-lactate, diamine oxidase, superoxide dismutase, malondialdehyde, glutathione and glutathione

	Group I	Group II	Group III	Group IV	Group V
D-lactate (mmol/L)	1.723 ± 0.106^{a}	4.236 ± 0.050^{c}	3.599 ± 0.181	3.152 ± 0.123^{e}	2.800 ± 0.129
DAO (U/L)	2.829 ± 0.438^{a}	18.925 ± 1.485^{c}	12.928 ± 1.544	10.198 ± 0.584^{e}	7.109 ± 0.590
SOD (U/mg protein)	65.002 ± 4.397^{a}	26.782 ± 1.979^{c}	35.396 ± 1.328	43.916 ± 1.720^{e}	53.066 ± 3.203
MDA (nmol/mg protein)	0.408 ± 0.054^{a}	1.253 ± 0.154^{c}	0.914 ± 0.108	0.672 ± 0.054^{e}	0.540 ± 0.029
GSH (μmol/L)	21.091 ± 0.452^{a}	7.235 ± 0.479^{c}	8.431 ± 0.537	10.504 ± 0.481^{e}	19.082 ± 0.455
GSSG (μmol/L)	2.974 ± 0.260^{a}	23.753 ± 2.895^{c}	12.795 ± 1.360	4.944 ± 0.207^{e}	3.537 ± 0.343

[a]$P < 0.05$ *vs* Group II; [c]$P < 0.05$ *vs* Group III; [e]$P < 0.05$ *vs* Group V. Group I: Sham-operation; Group II: Bile duct ligation (BDL); Group III: BDL + *L. plantarum*; Group IV: BDL + internal biliary drainage (IBD); Group V: BDL + IBD + *L. plantarum*. DAO: Diamine oxidase; SOD: Superoxide dismutase; MDA: Malondialdehyde; GSH: Glutathione; GSSG: Glutathione.
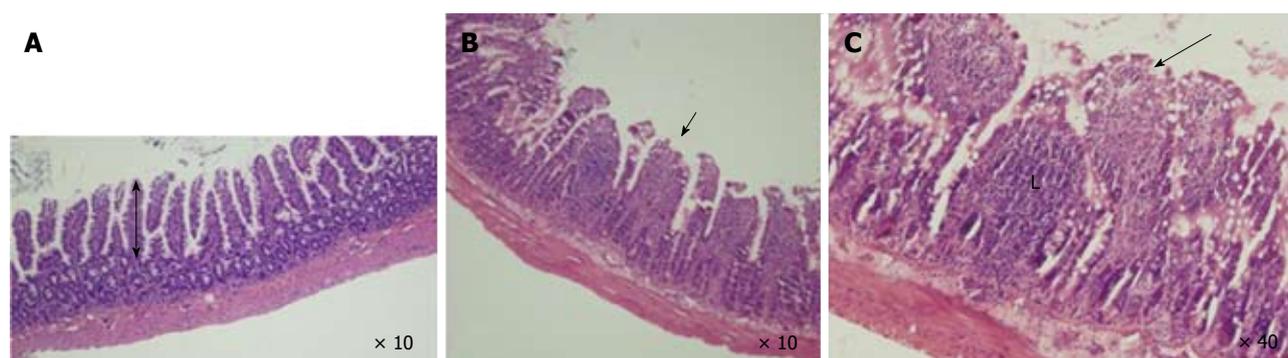
Figure 2 Light microscopic micrographs of samples stained with haematoxylin and eosin. A: Normal structure of villi (two-way arrow); B: Blunting of villi (short arrow); C: Existing subepithelial edema (longf arrow) and the lymphocyte and plasma cell infiltration (L). Images shown represent at least three regions observed on the same slide.

reduced the levels of GSSG in the BDL + IBD group (Table 3).

Intestinal mucosal SOD and MDA

Ileum mucosal MDA was significantly increased in BDL group compared with SHAM. Administration of *L. plantarum* significantly decreased the levels of MDA in the BDL + IBD group. The trend in ileum mucosal SOD levels among the groups was opposite to the results of MDA (Table 3).

Morphological studies

Specimens collected from the terminal ileum in the BDL group showed subepithelial edema and blunting of the villi, mostly located at the tip of the villi, with a large number of lymphocytes and plasma cells infiltrated in the intestinal mucosa (Figure 2). Under TEM, cell ultrastructure was disordered, with loss or disruption of microvilli and large dense secondary lysosomes that resembled partially degraded bacteria within the enterocyte cytoplasm. Inflated vacuolization of the cells, swollen mitochondria with partial or complete absence of interior cristae, disruption of desmosomes and formation of oedematous spaces, and expansion of endoplasmic reticulum were observed. Cells often showed serious damage to the plasma membrane and complete loss of junctional specialization between adjacent cells. Additionally, spherical and rod-shaped bacteria in the ileum were seen near enterocytes. These features are typical of prenecrotic and necrotic injury of the intestinal mucosa. However, in the BDL +

L. plantarum group, cells were aligned regularly, with less swelling of mitochondria, no expansion of the endoplasmic reticulum, while the morphology was nearly normal in the IBD + *L. plantarum* group (Figure 3).

Apoptosis in the intestinal mucosal epithelium

Apoptotic nuclei were significantly more abundant in the markedly atrophic villi in the BDL group than in the SHAM group. The apoptotic nuclei were mostly distributed at the top of villi (Figure 4A). Administration of *L. plantarum* significantly decreased the number of apoptotic nuclei in the BDL + IBD + *L. plantarum* group (Figure 4B).

Expression of PKC illustrated by immunocytochemistry

PKC appeared as brown spots in the perinuclear structure. Its expression was decreased in the BDL group compared with the SHAM group. Administration of *L. plantarum* significantly enhanced the expression of PKC in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Figure 5).

Effects of L. plantarum on TJ protein localization (fluorescence microscopy)

Confocal imaging was performed to assess the distribution of the TJs in each group. TJ-associated proteins were continuously distributed in bright green or red color along the membrane of the cells. The F-actin staining showed a continuous line at the cell borders and along the cytoskeleton. Their borders were very clear in the SHAM group, where TJ-associated proteins were present at the apical
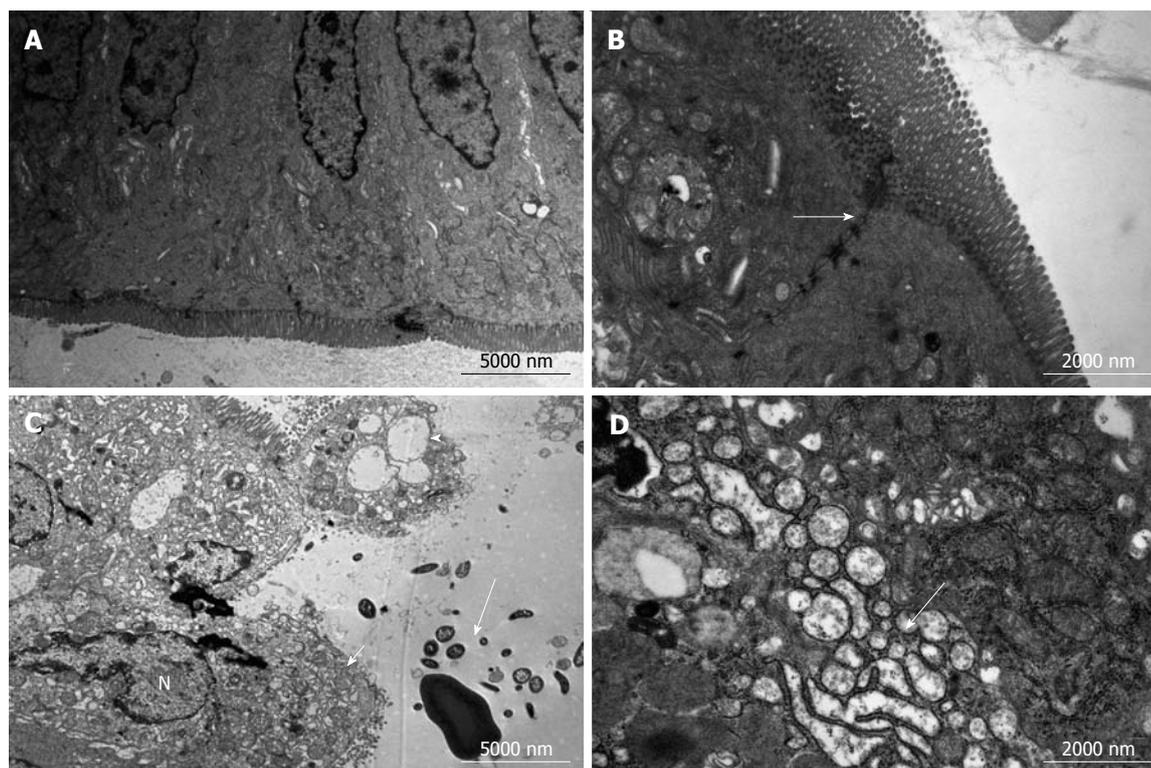
Figure 3 Ultrastructural assessment of enterocytes in the terminal ileum. A: Normal cell ultrastructure; B: The positions of tight junctions (arrow) and desmosomes; C: Enterocytes showed vacuolar degeneration (short arrow) and mitochondrial swelling, disruption of their microvilli (arrowhead), large dense secondary lysosomes, complete loss of the junctional specialization between adjacent cells (long arrow), and karyopyknosis (N); D: Expansion of the endoplasmic reticulum (arrow). Images shown represent at least three regions observed on the same slide. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ: BDL + IBD + *L. plantarum*.

intercellular borders in a belt-like manner, encircling the cells and delineating the cellular borders. In the BDL group, the fluorescence was dispersed and even became punctate, with loss of membrane fluorescence as against the uniform membrane staining in controls. Administration of *L. plantarum* enhanced the expression of TJ-associated proteins in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Figure 6).

Effects of L. plantarum on TJ and PKC protein levels (Western blotting)

Western blotting analyses were performed to determine the relative protein levels of the target proteins occludin, claudin-1, claudin-4, JAM-A, ZO-1 and PKC in the terminal ileum. The intensity of the whole-cell proteins was determined from ratios of the integrated intensity of the target protein bands to the integrated intensities of the β-actin bands in the same sample. Compared with samples obtained from rats in the SHAM group, levels of target proteins were decreased in protein extracts from ileal mucosal scrapings of rats subjected to BDL. Administration of *L. plantarum* significantly enhanced the expression of TJ-associated proteins in the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Figure 7).

Levels of mRNA in TJ and PKC determined by real-time PCR assays

Intragastric administration of *L. plantarum* resulted in

changes in the levels of occludin, ZO-1, claudin-1, claudin-4, JAM-A and PKC. This result raised the question whether these altered protein levels were a consequence of changes in mRNA levels. We, therefore, used real-time RT-PCR to determine the levels of mRNA in the terminal ileum in each group. The levels of mRNA of occludin, ZO-1, claudin-1, claudin-4, JAM-A, PKC and UGT1A1 were found significantly lower in the BDL group than in the SHAM group. Administration of *L. plantarum* significantly increased the mRNA levels of target proteins in both the BDL + *L. plantarum* and IBD + *L. plantarum* groups (Table 4).

Phosphorylation of occludin and ZO-1

We examined the phosphorylation status of occludin and ZO-1 using immunoprecipitation and immunoblotting assays. Occludin and ZO-1 were phosphorylated at serine residues. BDL lowered p-occludin and p-ZO-1 proteins levels compared with the SHAM group. Administration of *L. plantarum* improved the expression of the p-occludin and p-ZO-1 proteins from the terminal ileum in the BDL + *L. plantarum* and BDL + IBD + *L. plantarum* groups (Figure 8).

Effects of L. plantarum on PKC activity

As shown in Figure 9, PKC activity was significantly decreased in the BDL group compared with SHAM group. Intragastric administration of *L. plantarum* partly restored
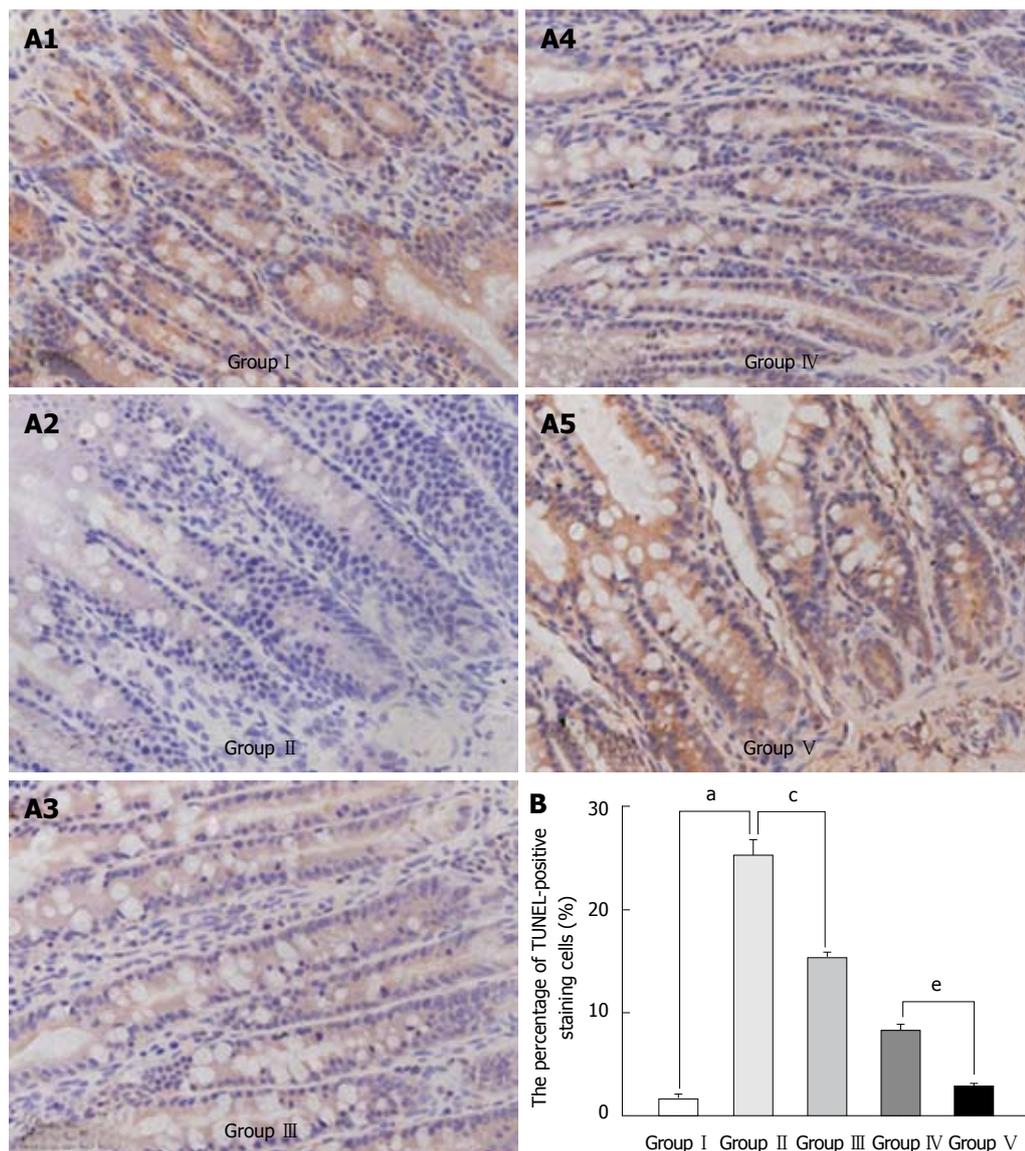
Figure 4 Effects of *Lactobacillus plantarum* on the apoptosis in the intestinal mucosal epithelium. A: Ileum sections from each group stained using the terminal deoxyuridine nick-end labeling (TUNEL) method. TUNEL-positive cells were stained dark brown. A significantly higher number of TUNEL-positive cells was detected in tissues from group Ⅱ animals compared with group Ⅲ; and the number of TUNEL-positive cells in group Ⅳ was higher than in group Ⅴ. Images shown represent at least three regions observed on the same slide; B: Statistical evaluation of effects of *Lactobacillus plantarum* (*L. plantarum*) on the apoptosis in the intestinal mucosal epithelium. Data in the bar graph represent mean ± SD of a minimum of three slides per group. [a]$P < 0.05$ Group Ⅰ *vs* Group Ⅱ; [c]$P < 0.05$ Group Ⅱ *vs* Group Ⅲ; [e]$P < 0.05$ Group Ⅳ *vs* Group Ⅴ. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *L. plantarum*; Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ: BDL + IBD + *L. plantarum*.

Table 4 Expression (mRNA) ratio (studied genes/β-actin) for tight junction and protein kinase C in terminal ileum tissues of each experimental group (mean ± SD)

Genes	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Occludin	2.5458 ± 0.2260	0.4881 ± 0.0426[a]	0.9792 ± 0.2066	1.4902 ± 0.0720[c]	1.8976 ± 0.1049
ZO-1	7.2420 ± 0.4025	0.9541 ± 0.1629[a]	1.4064 ± 0.1632	2.8843 ± 0.1641[c]	4.0727 ± 0.2059
Claudin-1	1.9751 ± 0.0615	0.0546 ± 0.0336[a]	0.4741 ± 0.0897	0.9092 ± 0.1295[c]	1.4793 ± 0.2119
Claudin-4	42.8680 ± 7.5291	0.3546 ± 0.0916[a]	5.3245 ± 1.1801	8.7719 ± 1.4659[c]	15.9592 ± 2.8815
JAM-A	3..3259 ± 0.3704	0.4712 ± 0.1107[a]	0.9456 ± 0.1101	1.6270 ± 0.2153[c]	2.1006 ± 0.1534
PKC	6.6958 ± 0.9349	1.7959 ± 0.2992[a]	2.8281 ± 0.3287	3.7178 ± 0.5110[c]	4.7235 ± 0.4958

[a]$P < 0.05$ *vs* Group Ⅲ; [c]$P < 0.05$ *vs* Group Ⅴ. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *L. plantarum*; Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ: BDL + IBD + *L. plantarum*. JAM-A: Junction adhesion molecule-A; PKC: Protein kinase C.
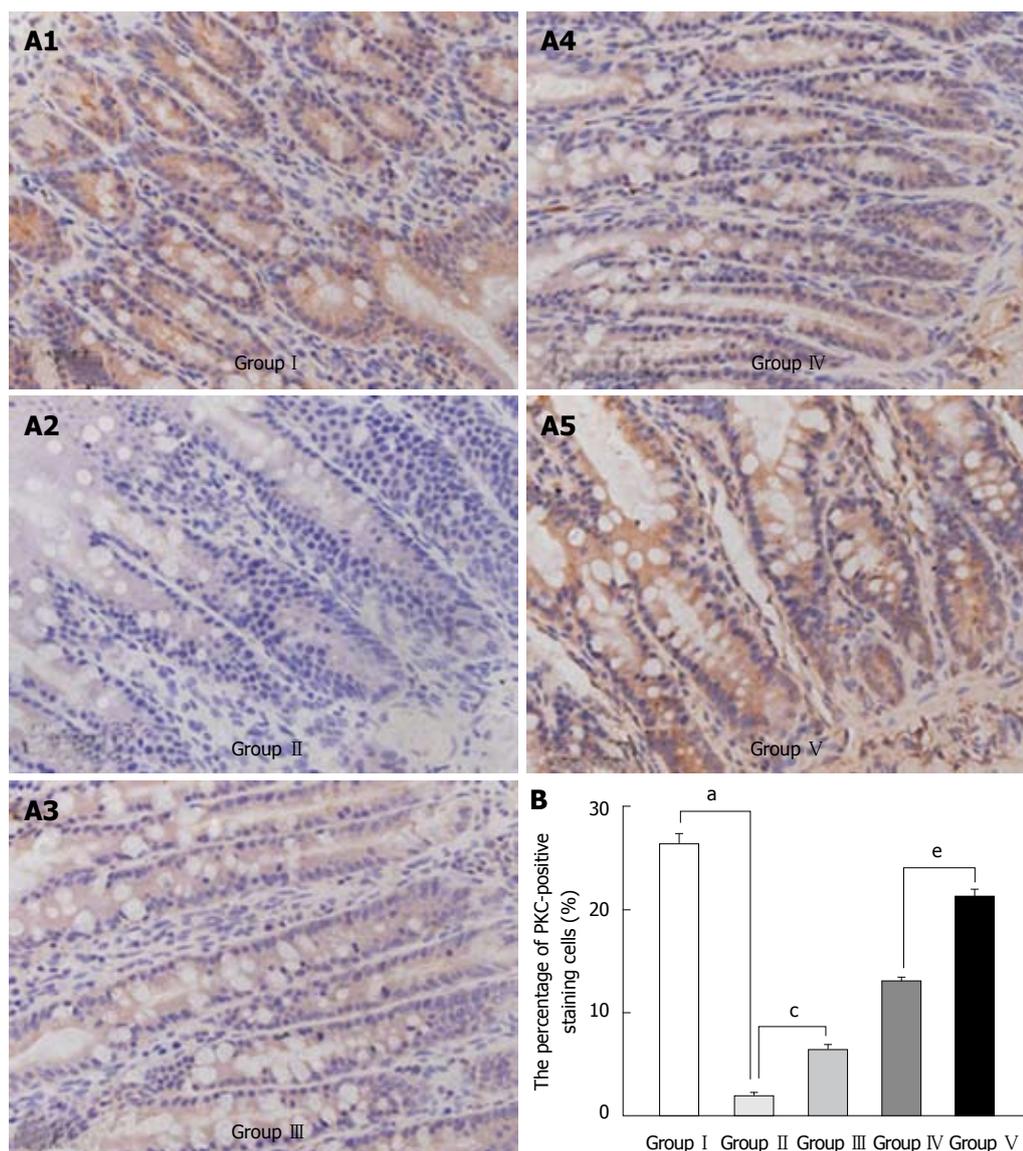
Figure 5 Effects of probiotics on the expression of protein kinase C in the mucosa of the terminal ileum. A: Probiotics effects on biliary obstruction-induced expression of protein kinase C (PKC) as determined by immunohistochemistry. Images shown are representative of at least three regions observed on the same slide; B: Statistical evaluation of effects of addition of probiotics (*Lactobacillus plantarum*) on the expression of PKC in the intestinal mucosal epithelium. Data in the bar graph represent mean ± SD of the three separate experiments. [a]$P < 0.05$ Group Ⅰ *vs* Group Ⅱ; [c]$P < 0.05$Group Ⅱ *vs* Group Ⅲ; [e]$P < 0.05$ Group Ⅳ *vs* Group Ⅴ. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ = BDL + IBD + *L. plantarum*.

PKC activity in both the BDL + *L. plantarum* and BDL + IBD + *L. plantarum* groups.

DISCUSSION

The present study demonstrated that oral administration of *L. plantarum* significantly reduced bilirubin, ALT and endotoxin levels in the systemic circulation in an experimental obstructive jaundice animal model with internal biliary drainage for 10 d. Moreover, oral *L. plantarum* administration to the rats in the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group not only reduced the serum endotoxin levels, but also substantially improved liver function. This result is consistent with the conclusions reported in previous literature[12].

In our study, oral *L. plantarum* administration also significantly decreased the serum DAO activity and *D*-lactate level in both the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group. These findings indicate that *L. plantarum* plays an important role in intestinal integrity. Previous *in vitro* studies have confirmed that probiotics exert direct protective effects in intestinal epithelial cell TJs *via* a PKC-kinase-dependent mechanism and inhibiting epithelial cell apoptosis in cell culture experiments[11,18,10]. The current experiments focused on *L. plantarum* effects, while several previous studies have reported the protective effects of other lactobacilli and probiotics. For example, Moorthy *et al*[27] reported that pretreatment with a combination of *Lactobacillus rhamnosus* (*L. rhamnosus*) and *Lactobacillus acidophilus* had a significant protective effect on TJ proteins (claudin-1 and occludin) in a *Shigella dysenteriae* 1 infection rat model. Khailova
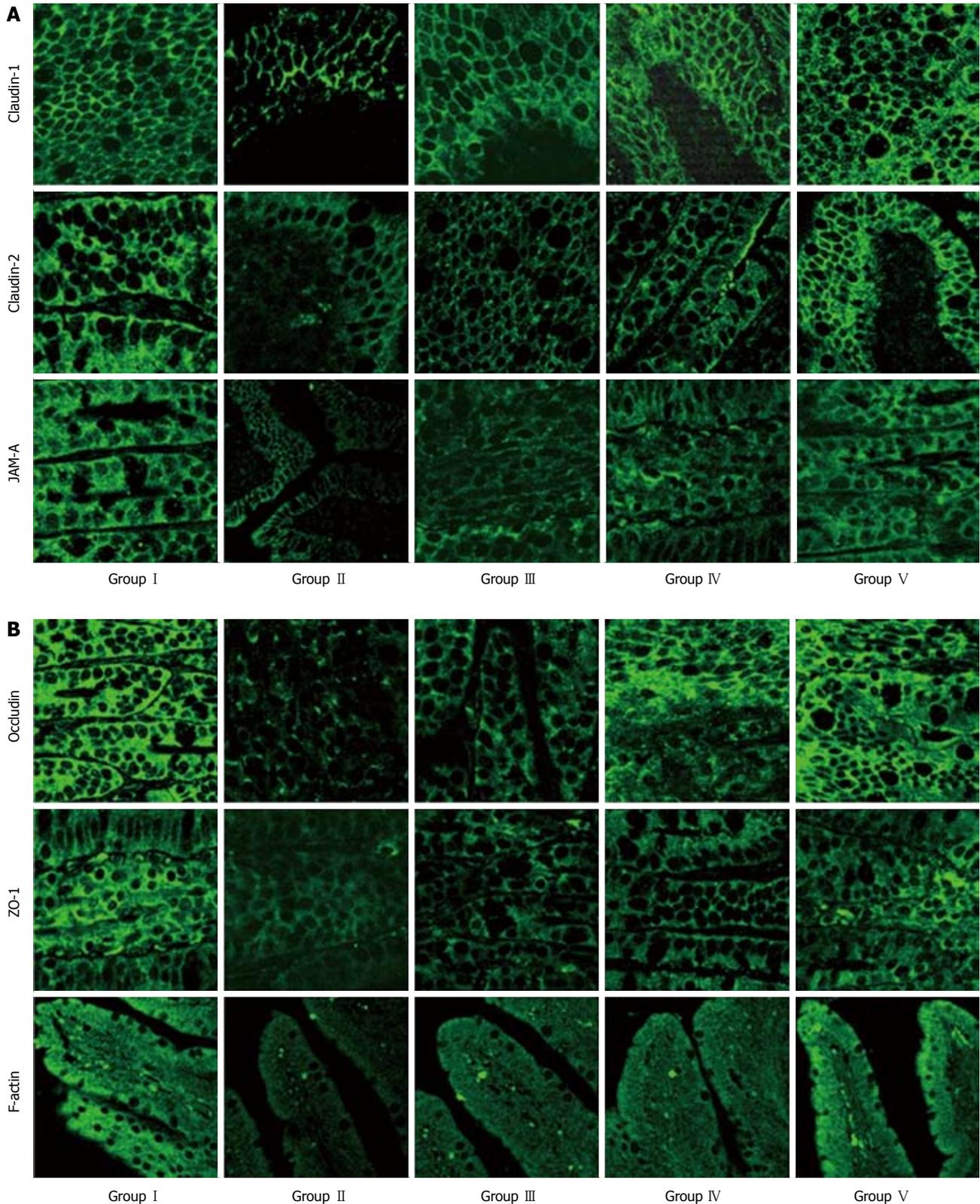
Figure 6 Immunofluorescence expression of claudin-1, claudin-4 and junction adhesion molecule-A (A) and occludin, ZO-1 and F-actin (B) in the mucosa of the terminal ileum. Images shown are representative of at least three regions observed on the same slide. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ = BDL + IBD + *L. plantarum*. JAM-A: Junction adhesion molecule-A.

et al[28] reported that *Bifidobacterium bifidum* improved intestinal integrity [composition of TJ and adherens junction (AJ) proteins] in a rat model of necrotizing enterocolitis.

Mennigen *et al*[29] found that the probiotic mixture VSL#3 protected the epithelial barrier by maintaining TJ protein expression and preventing apoptosis in a murine model of
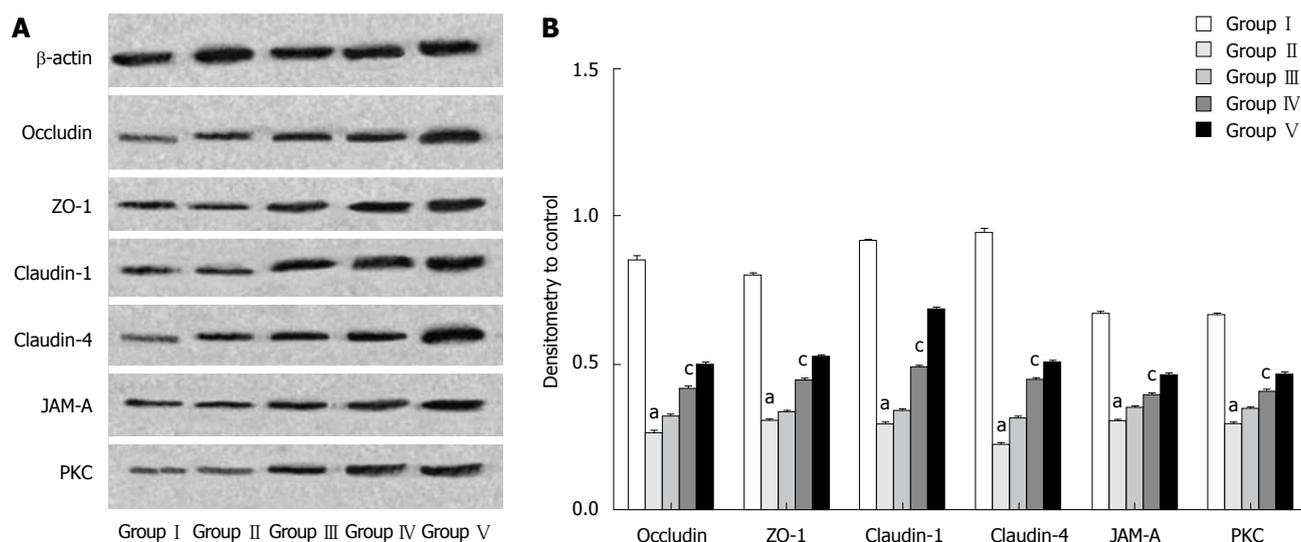
Figure 7 Effects of probiotics on the levels of tight junction proteins and protein kinase C proteins in the mucosa of the terminal ileum. A: Western blotting analysis of occludin, ZO-1, claudin-1, claudin-4, junction adhesion molecule (JAM)-A, and protein kinase C (PKC) proteins; B: Statistical evaluation of densitometric data that represent protein levels from the three separate experiments. [a]*P* < 0.05 *vs* Group Ⅲ; [c]*P* < 0.05 *vs* Group Ⅴ. Data are presented as relative band density ± SD. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ = BDL + IBD + *L. plantarum*.
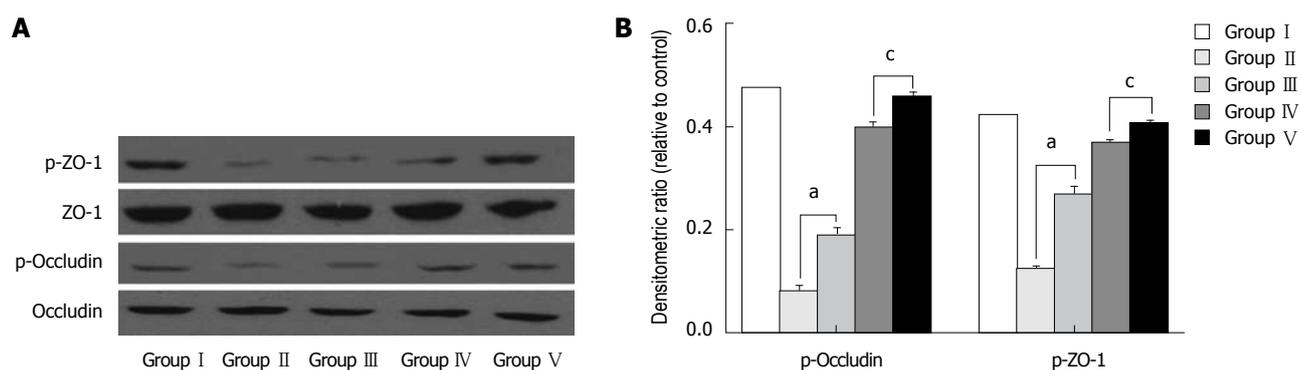
Figure 8 Serine phosphorylation of occludin, zonula occludens-1 in the terminal ileum. Tissue lysates were subjected to immunoprecipitation with the anti-occludin or zonula occludens-1 (ZO-1) antibody, followed by Western blotting analysis with antibodies against phosphoserine. A: p-Occludin and p-ZO-1 protein levels compared with untreated cells; B: Effects of addition of *Lactobacillus plantarum* on the expression of the p-occludin and p-ZO-1 proteins as shown by relative band density. Data in the bar graph represent mean ± SD of the three separate experiments. [a]*P* < 0.05 Group Ⅱ *vs* Group Ⅲ; [c]*P* < 0.05 Group Ⅳ *vs* Group Ⅴ. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ: BDL + IBD + *L. plantarum*.
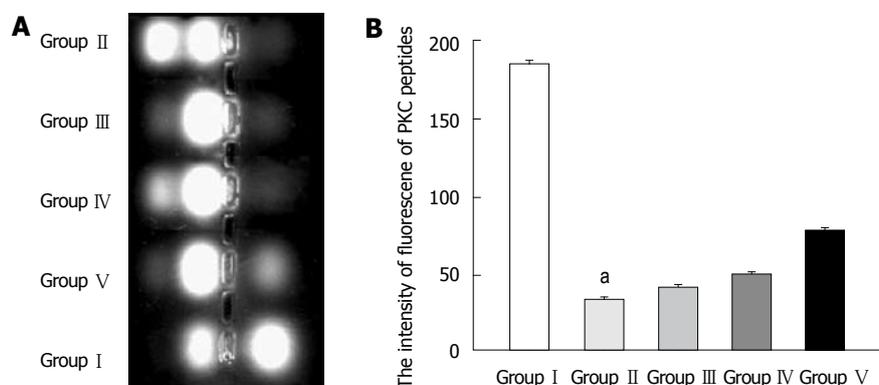
Figure 9 Effect of obstructive jaundice and probiotics on the activity of protein kinase C in the terminal ileum. A: A representative electrophoresis gel from the protein kinase C (PKC) activity assay; B: The averaged figures in each group of terminal ileum from the three separate experiments. [a]*P* < 0.05 *vs* each of the other four groups. Group Ⅰ: Sham-operation; Group Ⅱ: Bile duct ligation (BDL); Group Ⅲ: BDL + *Lactobacillus plantarum* (*L. plantarum*); Group Ⅳ: BDL + internal biliary drainage (IBD); Group Ⅴ: BDL + IBD + *L. plantarum*.

colitis. These studies support the findings that a number of probiotic agents have the protective effects in gastrointestinal tract as described in the current work.

Previous studies, including experimental models and clinical cases of biliary obstruction, have shown that disruption of intestinal barrier integrity in obstructive jaundice is associated with high intestinal oxidative stress, as evidenced by increased lipid peroxidation and oxidation of proteins, non-protein, and protein thiols[6,30]. Increased intestinal oxidative stress, a factor in cellular injury, may also play a critical role in regulating important cellular alterations of the intestinal mucosa in obstructive jaundice, such as increased apoptosis and altered TJ expression[5,31]. The results of this study strongly suggest that obstructive jaundice induces oxidative stress in the intestine.

We also found that apoptotic nuclei were significantly more abundant in markedly atrophic villi in the BDL group. Administration of *L. plantarum* significantly decreased apoptosis of the terminal ileum and improved the histology of the terminal ileum, which also was affected by obstructive jaundice. The protective effect of *L. plantarum* may be related to amelioration of oxidative stress. Previous studies of *L. rhamnosus* GG, a member of the same genus as *L. plantarum*, showed that this bacterium attenuated the H_2O_2-induced disruption of barrier function[18]. Our studies also revealed that administration of *L. plantarum* significantly reduced the levels of GSSG, MDA and SOD in rats of the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group.

Intestinal epithelial TJs prevent diffusion of potential injurious factors from the gastrointestinal lumen into the tissue. TJs located at the subapical aspect of the lateral membranes contain a large number of membrane-associated proteins, including occludin, JAM and claudins, which are responsible for forming the physical connections between cells that confer the basic barrier properties. Using immunohistochemistry and immunoblotting, previous studies have demonstrated that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with a regional loss of occludin expression in the intestinal epithelium[4,31]. Similarly，our study showed that levels of TJ-associated proteins such as occludin, ZO-1, claudin-1, claudin-4 and JAM-A were reduced in the intestinal epithelium, especially at the upper part of villi. These data support the conclusion that oral *L. plantarum* administration can enhance the expression of TJ-associated proteins.

A significant body of evidence indicates that PKC is involved in the regulation of the integrity of TJs and AJs. Recent studies have shown differences between Tyr-phosphorylation and Ser/Thr-phosphorylation of occludin. Tyr-phosphorylation of occludin is clearly associated with the disruption of TJs. Ser/Thr-phosphorylation may be required for the assembly of occludin into the TJs. PKC-ζ prevents oxidant-induced iNOS upregulation and protects microtubules and gut barrier integrity[32]. Thus, PKC-ζ appears to be an endogenous stabilizer of the microtubule cytoskeleton and of intestinal barrier function against oxidative injury[33].

The probiotic bacterium *L. plantarum* has been shown to improve intestinal barrier function in a range of experimental models of colitis, pancreatitis, liver injury and biliary obstruction[8,34-37]. Recent studies have shed some light on the mechanisms involved in the beneficial effects of probiotics in the gastrointestinal tract. PKC activity may be involved in epidermal growth factor-mediated protection of the intestinal epithelial barrier function against oxidative stress[38]. Seth *et al*[18] suggested that PKCβI activation may be one of the initial events in the probiotic-mediated protection of TJs and AJs. PKCε may play a role in the downstream events of the signaling pathway involved in this process. TJ-protein phosphorylation mediated by PKC may be related to the mechanism of protection by *L. plantarum* in obstructive jaundice. Previous studies have shown that phosphorylation is a key mechanism for regulating the biological function of TJ proteins. Highly phosphorylated occludin molecules are selectively concentrated at TJs, whereas non- or less phosphorylated occludin molecules are localized in the cytoplasm[39].

To determine whether PKC mediates the disruption of the intestinal barrier in obstructive jaundice, we examined the phosphorylation status of occludin and ZO-1 using Western blotting analysis with antibodies against phosphoserine. We found that obstructive jaundice decreased p-occludin and p-ZO-1 protein levels compared with sham-operation. Our study also demonstrated that obstructive jaundice resulted in a significant decrease in PKC activity. Co-incubation with *L. plantarum* partly restored PKC activity and increased phosphorylation of serine residues on TJ proteins in both the BDL + *L. plantarum* group and the BDL + IBD + *L. plantarum* group. Phosphorylation of these proteins occurred on Ser residues that have been described as substrates for PKC activity[40-42]. Our results suggest that the PKC pathway is involved in the process of *L. plantarum*-induced TJ redistribution.

In conclusion, administration of probiotics before and after operation in rats with experimental obstructive jaundice can substantially protect the gut barrier. The protective mechanisms of probiotics are associated with decreased intestinal epithelial cell apoptosis, reduction of oxidative stress, and protection of intestinal mucosal TJs. *L. plantarum* can prevent TJ disruption in biliary obstruction by activating the PKC pathway.

COMMENTS

Background

Biliary tract surgery in patients with obstructive jaundice is associated with a high morbidity and mortality rate, and obstructive jaundice increased gut permeability and bacterial translocation. *Lactobacillus plantarum* (*L. plantarum*) has been shown to have beneficial effects on intestinal barrier function. Protein kinase C (PKC) plays a crucial role in the mediation of intestinal epithelial tight junction (TJ) proteins, and *L. plantarum* may prevent TJ disruption in biliary obstruction by activating the PKC pathway. However, there had been few studies about the mechanism for the protective effect of probiotics on the intestinal barrier in obstructive jaundice. This study focused on the effects of *L. plantarum* on the intestinal mucosal barrier, oxidative stress, epithelial TJ-protein structure

and phosphorylation, especially its impact on the expression and activity of PKC.

Research frontiers

Previous *in vitro* studies have confirmed that probiotics could protect intestinal epithelial cell TJs *via* a PKC-kinase-dependent mechanism and inhibit epithelial cell apoptosis in cell culture experiments. TJ-protein phosphorylation mediated by PKC may be related to the protective effects of *L. plantarum* in obstructive jaundice.

Innovations and breakthroughs

The administration of *L. plantarum* before and after operation in rats with experimental obstructive jaundice could substantially protect the gut barrier. Protective mechanisms of *L. plantarum* are associated with decreased intestinal epithelial cell apoptosis, reduction of oxidative stress, and protection of intestinal mucosal TJs. *L. plantarum* can prevent TJ disruption in biliary obstruction by activating the PKC pathway.

Applications

By understanding the mechanism and effects of *L. plantarum* on the intestinal mucosal barrier, this study may represent a future strategy in the treatment of patients with obstructive jaundice.

Peer review

This is a very well done experimental study for evaluating the effect of *L. plantarum* on the intestinal mucosal barrier, oxidative stress, epithelial TJ protein structure and phosphorylation, with special regard to its impact on the expression and activity of PKC in experimental obstructive jaundice.

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BRIEF ARTICLE

Diagnostic and therapeutic direct peroral cholangioscopy using an intraductal anchoring balloon

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Abstract

AIM: To report our experience using a recently introduced anchoring balloon for diagnostic and therapeutic direct peroral cholangioscopy (DPOC).

METHODS: Consecutive patients referred for diagnostic or therapeutic peroral cholangioscopy were evaluated in a prospective cohort study. The patients underwent DPOC using an intraductal anchoring balloon, which was recently introduced to allow consistent access to the biliary tree with an ultraslim upper endoscope. The device was later voluntarily withdrawn from the market by the manufacturer.

RESULTS: Fourteen patients underwent DPOC using the anchoring balloon. Biliary access with an ultraslim upper endoscope was accomplished in all 14 patients. In 12 (86%) patients, ductal access required sphincteroplasty with a 10-mm dilating balloon. Intraductal placement of the ultraslim upper endoscope allowed satisfactory visualization of the biliary mucosa to the level of the confluence of the right and left hepatic ducts in 13 of 14 patients (93%). Therapeutic interventions by DPOC were successfully completed in all five attempted cases (intraductal biopsy in one and DPOC guided laser lithotripsy in four). Adverse events occurred in a patient on immunosuppressive therapy who developed an intrahepatic biloma at the site of the anchoring balloon. This required hospitalization and antibiotics. Repeat endoscopic retrograde cholangiopancreatography 8 wk after the index procedure showed resolution of the biloma.

CONCLUSION: Use of this anchoring balloon allowed consistent access to the biliary tree for performance of diagnostic and therapeutic DPOC distal to the biliary bifurcation.

INTRODUCTION

The value of cholangioscopy for diagnosis and treatment of biliary disorders has been well established[1]. Diagnostic and therapeutic direct peroral cholangioscopy (DPOC) using an ultraslim upper endoscope offers significant advantages over ductoscopy using dedicated cholangioscopes[2]. The ultraslim endoscope uses a single-operator platform, provides high-definition digital image quality, allows simultaneous irrigation and therapy,

is not fragile, and has a larger working channel enabling enhanced diagnostic sampling and therapeutic interventions. However, DPOC in its current state has significant limitations. Initial free biliary cannulation is cumbersome, time consuming, and associated with a significant failure rate. Even when access is obtained, intraductal maneuverability and stability may be compromised by looping of the endoscope in the stomach or duodenum. To address these technical challenges, a prototype biliary anchoring balloon has been designed. Feasibility of this system in an animal model has been assessed but human reports are lacking[2].

We performed a study in 14 patients to assess utility of this device for diagnosis and treatment of various biliary disorders prior to voluntary device withdrawal by the manufacturer. We report our experience using this device for ductal access in all 14 patients.

MATERIALS AND METHODS

Study aims and patients
This was a prospective cohort study of consecutive patients referred to our tertiary care center for endoscopic retrograde cholangiopancreatography (ERCP) and peroral cholangioscopy. The primary aim of the study was to assess the ability to gain access to the bile duct with an ultraslim upper endoscope and visualize the extrahepatic biliary mucosa to the level of the confluence of the left and right hepatic ducts. The secondary aim was to examine the feasibility of performing therapeutic procedures through the endoscope while maintaining intraductal access. This study was approved by The Cleveland Clinic Institutional Review Board. The inclusion criteria were presence of biliary pathology and ability of the patients to give informed consent. The exclusion criteria were coagulopathy, suspicion for acute ascending cholangitis, latex allergy, and biliary ductal diameter < 6 mm.

Anchoring balloon
The anchoring balloon used in this study (Cook Medical, Winston-Salem, NC, United States) had five components. A short stylet located at the most proximal end of the device was used to occlude the air channel after the balloon was inflated. This prevented deflation of the balloon after removal of the handle. Immediately distal to the stylet, there was a handle, which could be detached and removed to allow backloading of the device into an ultraslim upper endoscope while maintaining balloon inflation. The device had a 300-cm long, 4-French catheter with a nitinol stiffening core. At the distal end of the catheter, there was a latex balloon that could be inflated to 15 mm and used as an intraductal anchoring point. A radiopaque loop tip was located at the most distal end of the device and allowed positioning of the balloon in the desired duct under fluoroscopic guidance over a guidewire (Figure 1A).

Cholangioscopy procedure
All procedures were performed by an experienced endos-

copist, under monitored anesthesia care sedation with the patients in the prone position. Endoscopic sphincterotomy was or had been performed previously prior to direct peroral cholangioscopy in all patients. Balloon sphincteroplasty to 10 mm was performed as needed. After completion of ERCP, a 0.889-mm guidewire was placed in one of the intrahepatic ducts. The anchoring balloon was then directed into that intrahepatic duct by placing the loop end of the device over the guidewire. Location of the guidewire and the anchoring balloon was verified by fluoroscopy (Figure 1B). The balloon was then inflated with air to anchor it within the duct. A gentle pull on the balloon catheter confirmed ductal anchoring. If the pull on the catheter led to dislodgment of the balloon, it was deflated and the procedure was repeated to reposition the balloon in another intrahepatic duct followed by inflation of the balloon and pulling to confirm anchoring. Once anchoring of the balloon was confirmed, the air channel of the device was covered with the stylet to keep the balloon inflated. The proximal handle was then detached from the catheter and removed. This was followed by removal of the duodenoscope and the guidewire, leaving the inflated balloon and its catheter behind. An ultraslim upper endoscope was backloaded over the catheter and advanced into the bile duct to the level of the confluence of the right and left hepatic ducts (Figure 1C). The anchoring balloon was then removed in most cases. The bile duct was irrigated through the accessory channel of the ultraslim endoscope with sterile saline solution, followed by slow withdrawal of the endoscope, allowing systematic inspection of the biliary tree. Air insufflation was not used, to avoid potential complications. Routine antibiotic prophylaxis was not administered. The ultraslim endoscopes used in this study included GIF-XP160 (outer diameter 5.9 mm), GIF-XP180 (outer diameter 5.5 mm), and GIF-N180 (outer diameter 4.9 mm) (Olympus Corporation, Center Valley, PA United States). All endoscopes had an instrument channel with an inner diameter of 2 mm.

Statistical analysis
Values are presented as mean (range) or frequency (percentage). R version 2.4.1 software (The R Foundation for Statistical Computing, Vienna, Austria) was used to perform all the analyses.

RESULTS

Patients and indications
Fourteen consecutive patients underwent DPOC using the new anchoring balloon. The mean age of the patients was 65 years (range: 30-92 years). Nine (64%) patients were female. The indications for the procedure, DPOC findings and final diagnosis are presented in Table 1.

Biliary access
Biliary access with an ultraslim upper endoscope was accomplished in all patients. In 12 of 14 (86%) patients, biliary access required sphincteroplasty with a 10-mm
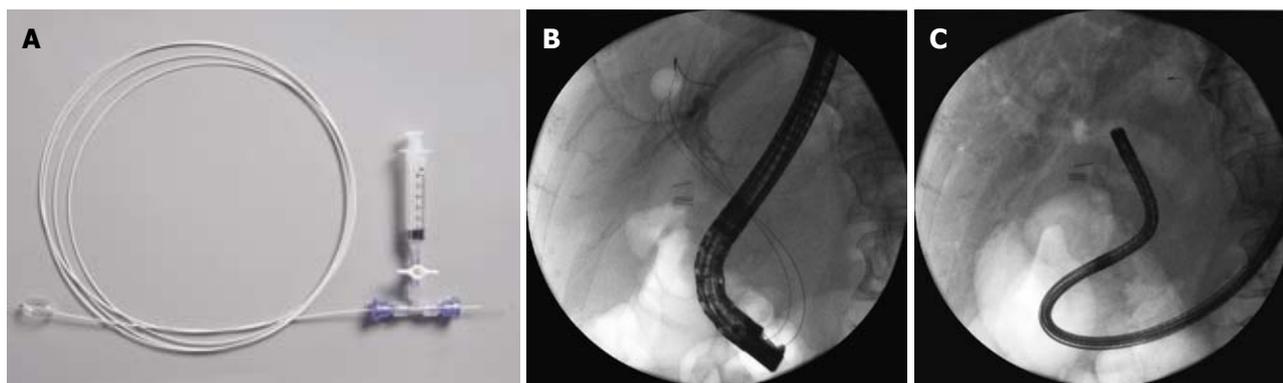
Figure 1 *Ex vivo* and *in vivo* views of the anchoring balloon. A: Overall view of the anchoring balloon; B: Fluoroscopic view of an inflated anchoring balloon that has been passed over a guidewire and anchored in one of the intrahepatic bile ducts; C: Fluoroscopic view of an ultraslim upper endoscope that has been backloaded over the catheter of an inflated anchoring balloon and advanced into the bile duct to the level of the confluence of the right and left hepatic ducts.

Table 1 Indications for diagnostic and therapeutic direct peroral cholangioscopy

Indication	DPOC diagnosis	Final diagnosis	Performance of sphincteroplasty	Largest bile duct diameter (mm)
Obstructive jaundice	Extrinsic stricture	Pancreatic cancer	Yes	18
Obstructive jaundice	Extrinsic stricture	Chronic pancreatitis	No	12
Suspicion for missed stones by ERCP	Stone in the main bile duct	Missed stone by ERCP	Yes	17
Difficult-to-remove bile duct stone	Large CBD stone	DPOC-guided laser lithotripsy followed by stone removal	Yes	11
Biliary stricture in a patient with PSC	Benign stricture	Benign PSC stricture	Yes	10
Biliary filling defect on MRI	Intraductal tumor	Intrahepatic cholangiocarcinoma with intraductal extension	Yes	10
Suspicion for missed stone(s) by ERCP	No stones found		Yes	8
Abnormal LFTs in a post liver transplantation patient	Missed stones during ERCP	Missed stones during ERCP	No	11
Surveillance after biliary polypectomy	Complete removal of the polyp		Yes	9
Difficult-to-remove bile duct stones	Four stones in the main bile duct	DPOC-guided laser lithotripsy followed by stone removal	Yes	13
Suspected polyp in CBD	Stone in CBD	CBD stone	Yes	20
Suspicion for missed stones by ERCP	No stones found		Yes	15
Difficult-to-remove bile duct stone	Large CBD stones	DPOC-guided laser lithotripsy followed by stone removal	Yes	22
Difficult-to-remove bile duct stone	Large stone above anastomotic stricture	DPOC-guided laser lithotripsy followed by stone removal	Yes	16

DPOC: Diagnostic and therapeutic direct peroral cholangioscopy; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct; LFTs: Liver function tests; PSC: Primary sclerosing cholangitis; MRI: Magnetic resonance imaging.

dilating balloon over a guidewire. In 13 of 14 (93%) patients, the bile duct mucosa from the bifurcation to the ampulla could be well visualized. In one patient, the common hepatic duct could not be examined due to a sigmoid-shaped main bile duct that prevented passage of the endoscope proximal to the common bile duct. In this patient, only the mucosa of the common bile duct was inspected.

Biliary intervention

Therapeutic measures were attempted in five patients. Four patients underwent removal of difficult-to-remove bile duct stones and one patient had intraductal biopsy.

The four patients with difficult-to-remove stones had at least one prior unsuccessful attempt at stone extraction by ERCP with sphincterotomy, sphincteroplasty, and mechanical lithotripsy. In all cases, laser lithotripsy through

the ultraslim upper endoscope successfully fragmented the stones, with subsequent removal of the fragments (Figure 2). In one of these cases, however, instability of the ultraslim upper endoscope after removal of the anchoring balloon, required passage of the laser probe (SlimLine GI, Lumenis, Santa Clara, CA, United States) into the bile duct alongside the anchoring balloon, while keeping the balloon inflated in one of the intrahepatic ducts to maintain access. Nonetheless, the procedure was successful with excellent views of the biliary mucosa and performance of laser lithotripsy under direct vision with fragmentation and subsequent removal of the stone fragments.

In one patient, DPOC was performed to evaluate an ill-defined filling defect at the biliary confluence with intrahepatic ductal dilatation seen on magnetic resonance imaging. DPOC successfully visualized the filling defect
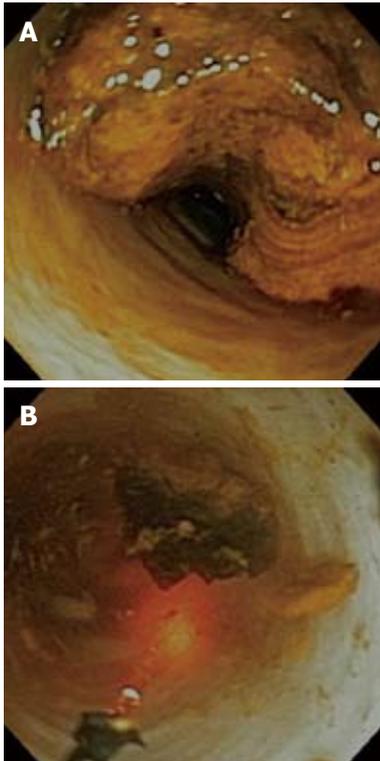
Figure 2 Cholangioscopic view of a large stone. A: The stone could not be removed during prior endoscopic retrograde cholangiopancreatography. A plastic stent was placed and the patient was referred for cholangioscopy-guided laser lithotripsy. Impression of the plastic stent in the stone is clearly visible; B: Cholangioscopic view of the same stone after laser lithotripsy. The laser probe is seen in the left lower corner of the picture.

Figure 3 Cholangioscopic image of a tumor in the proximal common hepatic duct. The anchoring balloon catheter is seen in the left lower corner of the picture.

Figure 4 Biloma at the site of the anchored balloon within an intrahepatic bile duct.

and showed it to be a tumor projecting into the bile duct lumen (Figure 3). Biopsies of the tumor were obtained through the ultraslim upper endoscope using a regular pediatric forceps (EndoJaw FB-221K; Olympus Corporation, Tokyo, Japan) and showed necrotic tissue. Follow-up imaging studies showed growing intraductal tumor suggesting cholangiocarcinoma.

Adverse events
One patient who had undergone orthotopic liver transplantation 2 years prior to the procedure, and was on immunosuppressive therapy, developed intrahepatic biloma at the site where the balloon was anchored within the duct (Figure 4). She underwent biliary stenting to assure ductal drainage. She also was given an intravenous dose of antibiotics during the procedure and discharged home on oral antibiotics and outpatient follow-up. However, 2 d after discharge she developed fever. She was admitted to the hospital and treated with intravenous antibiotics followed by 2 wk of oral antibiotics after discharge. Repeat ERCP 8 wk after the index procedure showed resolution of the biloma.

DISCUSSION
Despite its many advantages, DPOC is rarely performed in nonacademic settings. The biggest disadvantage of DPOC has been the difficult and time-consuming task

of bile duct cannulation with an upper endoscope, often ending in failure. There are several published reports in the endoscopic literature with innovative suggestions on how to achieve this task. Introduction of the endoscope over a guidewire, through a regular overtube, or with the help of a double-balloon overtube are some of the suggestions[3-6]. However, despite use of these accessories, failure rate remains high[7].

Different variations of inflatable balloons used as an anchor within the biliary tree have therefore been introduced for easier access[2,8]. Using such a device, we were able to gain access to the bile duct with an ultraslim upper endoscope in all cases and obtain high-quality images of the biliary mucosa. We were also able to perform necessary interventions such as shock wave lithotripsy or targeted biopsy in all cases in which therapy was attempted. In one of the therapeutic cases, however, the instability of the ultraslim endoscope upon removal of the balloon could only be remedied by keeping the anchoring balloon in position.

Although this device performed well for allowing access to the bile duct for performance of DPOC and assessment and therapy of disorders of the distal biliary system, we found it difficult to maneuver the endoscope and gain access to the ducts proximal to the bifurcation after deflation and removal of the anchoring balloon.

This was mainly due to looping of the endoscope in the stomach or duodenum after removal of the balloon.

The anchoring balloon used in this study was voluntarily withdrawn from the market by the manufacturing company, reportedly because of possible increased risk of air embolism[9]. The possible increased risk of air embolism is probably due to the ability of the ultraslim upper endoscopes to insufflate the biliary tree with air, while at the same time blocking the escape rout of the insufflated air. In this study, we used irrigation with saline rather than air to distend the bile duct and visualize the mucosa of the biliary tree.

One of the patients developed a biloma at the anchoring site of the balloon. Although she had a full recovery with antibiotic therapy, ductal damage at the anchoring site may be another potential adverse event associated with use of anchoring balloons.

One of the main limitations of this study was the small number of patients. However, the primary objective of this study was to assess performance of this device in a limited number of patients. Another limitation was performance of the procedures in a tertiary care referral setting by an endoscopist proficient in all forms of peroral and percutaneous cholangioscopy. This may limit extrapolation of the results to other settings.

In conclusion, our experience suggests that anchoring balloons are effective for consistent access to the bile duct with an ultraslim upper endoscope for performance of diagnostic and therapeutic DPOC distal to the confluence of the right and left hepatic ducts. However, we urge caution with widespread use of anchoring balloons until more information on potential adverse effects is available.

COMMENTS

Background
Direct peroral cholangioscopy (DPOC) in its current state has significant limitations. Initial free biliary cannulation is cumbersome, time consuming, and associated with a significant failure rate. Even when access is obtained, intraductal maneuverability and stability may be compromised by looping of the endoscope in the stomach or duodenum.

Research frontiers
To address the technical challenges associated with DPOC, a prototype biliary anchoring balloon has been designed. Feasibility of this system in an animal model has been assessed but human reports are lacking. This is the first study assessing utility of this prototype anchoring balloon for diagnosis and treatment of various biliary disorders.

Innovations and breakthroughs
Despite its many advantages, DPOC is rarely performed in nonacademic

settings. The biggest disadvantage of DPOC has been the difficult and time-consuming task of bile duct cannulation with an upper endoscope, often ending in failure. There are several published reports in the endoscopic literature with innovative suggestions on how to achieve this task. Introduction of the endoscope over a guidewire, through a regular overtube, or with the help of a double-balloon overtube are some of the suggestions. However, despite use of these accessories, the failure rate remains high. Using an anchoring balloon, the authors were able to gain access to the bile duct with an ultraslim upper endoscope in all cases and obtain high-quality images of the biliary mucosa. They were also able to perform necessary interventions such as shock wave lithotripsy or targeted biopsy in all cases in which therapy was attempted.

Applications
This study suggests that anchoring balloons are effective for consistent access to the bile duct with an ultraslim upper endoscope for performance of diagnostic and therapeutic DPOC distal to the confluence of the right and left hepatic ducts.

Peer review
The authors report clinical experiences of DPOC by using a novel balloon catheter as an assisting accessory. This is a well-written article. However, it is necessary that the authors offer more detailed descriptions and revise several major and minor points.

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BRIEF ARTICLE
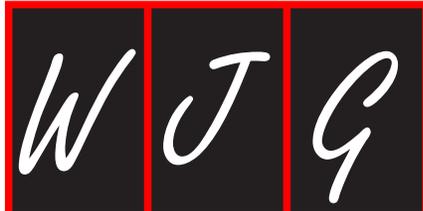
Age distribution, polyps and rectal cancer in the Egyptian population-based cancer registry

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Abstract

AIM: To describe the clinical and epidemiologic profiles of the disease and to compare the findings with those generated from the previous hospital-based studies.

METHODS: The Gharbiah cancer registry is the only population-based cancer registry in Egypt since 1998. We analyzed the data of all colorectal cancer patients included in the registry for the period of 1999-2007. All medical records of the 1364 patients diagnosed in Gharbiah during the study period were retrieved and the following information abstracted: age, residence, diagnosis date, grade, stage, topology, clinical characteristics, and histology variables. Egyptian census data for 1996 and 2006 were used to provide the general population's statistics on age, sex, residence and other related demographic factors. In addition to age- and sex-specific incidence rate analyses, we analyze the data to explore the incidence distribution by rural-urban differences among the 8 districts of the province. We also compared the incidence rates of Gharbiah to the rates of the Surveillance Epidemiology and End Results (SEER) data of the United States.

RESULTS: Over the 9 year-period, 1364 colorectal cancer cases were included. The disease incidence under age 40 years was relatively high ($1.3/10^5$) while the incidence in the age groups 40 and over was very low ($12.0/10^5$, $19.4/10^5$ and $21.2/10^5$ in the age groups 40-59 years, 60-69 years and > 70 years, respectively). The vast majority of tumors (97.2%) had no polyps and 37.2% of the patients presented with primary lesions in the rectum. Colorectal cancer was more common in patients from urban (55%) than rural (45%) areas. Regional differences in colon and rectal cancer incidence in the 8 districts of the study province may reflect different etiologic patterns in this population. The registry data of Egypt shows a slightly higher incidence of colorectal cancer than the United States in subjects under age 40 years. The results also shows significantly lower incidence of colorectal cancer in subjects over age 40 years compared to the same age group in the United States SEER.

CONCLUSION: Low rate of polyps, low incidence in older subjects, and high rate of rectal cancer in Egypt. Future studies should explore clinical and molecular disease patterns.

INTRODUCTION

Colorectal cancer is a common cancer worldwide. In 2008, Globocan estimated there were 663 000 new cases in men and 571 000 new cases in women[1,2]. With a combined 608 000 deaths worldwide, colorectal cancer represents the fourth most common cause of cancer-related mortality. There is a wide variability in the incidence rates of colorectal cancer, with most incident cases occurring in developed countries[1]. Incidence rates of colorectal cancer for men range from $4.1/10^5$ in Karunagappally, India, to $59.1/10^5$ in the Czech Republic. In women, incidence rates range from $3.6/10^5$ in Karunagappally, India, up to $39.5/10^5$ in New Zealand[2]. Rates for the United States are $34.1/10^5$ for men and $25/10^5$ for women[1].

Westernization is often associated with higher incidence rates of colorectal cancer. Diet and lifestyle factors are implicated risk factors for the disease. Fruit and vegetable-deficient diet, calorie-dense foods, physical inactivity, obesity, and smoking increase the risk for developing colorectal cancer[2]. While developing countries historically have a low rate of colorectal cancer, the transition to a more Western diet has been associated with increasing rates of disease[3,4].

Our previous hospital-based studies in Egypt showed low incidence of colorectal cancer and high proportion of young-onset disease[5]. In comparing rates of Egyptian colorectal cancer to the Surveillance Epidemiology and End Results Program (SEER) of the United States, Egypt had higher rates up to age 30-34 years, at which point Egyptian rates level out while United States rates increase sharply[5]. Our studies also revealed low rate of polyps, high proportion of rectal cancers among the colorectal cancer tumors, and lack of molecular characteristics of hereditary non-polyposis colorectal cancer (HNPCC) or young-onset or sporadic colorectal cancer in the United States[5-7]. Our studies have also shown intense environmental exposures such as organochlorine pesticide levels, however, these environmental and genetic factors were not related to the young or old onset of the disease[8].

Limitations of the previous studies on colorectal cancer in Egypt included their hospital-based nature and relatively small sample size. Therefore, with the establishment of Egypt's only population-based cancer registry and availability of complete data from 1999-2007, it was intriguing to investigate the epidemiologic and clinical profiles of colorectal cancer and examine the previous findings based on the new population-based registry data.

MATERIALS AND METHODS

The Gharbiah province is in in the center of the Nile delta region about 90 km north of Cairo with a population of about 4 million individuals[9]. It has a male:female ratio of 1.02:1 and age structure approximately equivalent to that of the rest of Egypt[10].

The Gharbiah population-based cancer registry, the only population-based registry in Egypt was founded in 1998[9]. It actively collects information on all cancer cases in the province from 3 main cancer hospitals; Tanta Cancer Center, the Gharbiah Cancer Society Hospital, and the Tanta University Hospital. In addition, information on cancer patients is collected from all private clinics and laboratories throughout the province[9]. The registry receives support and training from the National Cancer Institute (NCI) in Bethesda, Maryland through the Middle East Cancer Consortium (MECC). Technical support, training, and quality control of data are periodically conducted by Rollins School of Public Health, the International Agency for Research on Cancer, and NCI to ensure the high quality of the registry data[9,11].

All colorectal cancers diagnosed from 1999 to 2007 were included in the study. Medical records of 1364 patients were retrieved and the following information abstracted: age, residence, diagnosis date, grade, stage, topology, clinical characteristics and histology variables. Egyptian census data were used to provide the general population's statistics on age, sex, residence, and other related demographic factors. The 1364 cases included in this study over the 9 years from 1999-2007 average out to about 150 cases a year, which is in keeping with the registry's previous preliminary reports[9,12,13].

The student's *t*-test was used to determine the significance of differences in mean values of the study variables. A χ^2 test for independence was used to determine the significance of differences in frequency distributions and proportions of variables. The cut-off value for statistical significance was P value $= 0.05$. SAS version 9.2 (SAS Institute, Cary, NC) was used.

RESULTS

The results of the study provide a comprehensive profile of colorectal cancer in this population. This profile sheds some lights on the epidemiologic, clinical, and geographic distribution of colorectal cancer in this population. The results also demonstrate the differences in colorectal cancer incidence between the population-based registry of Gharbiah and the SEER registry of the United States.

A total of 1364 cases of colorectal cancer were included in registry from 1999-2007. Table 1 shows the summary characteristics for the colorectal cancer patients.

Table 1 Characteristics of study population and cancer patterns in Gharbiah, Egypt (1999-2007)	
Variable	*n* (%)
Total cases	1364 (100.0)
Gender	
Male	737 (54.0)
Female	627 (46.0)
Residence	
Urban	752 (55.1)
Rural	612 (44.9)
Year of diagnosis	
1999	135 (9.9)
2000	149 (10.9)
2001	144 (10.6)
2002	154 (11.3)
2003	151 (11.1)
2004	137 (10.0)
2005	161 (11.8)
2006	171 (12.5)
2007	162 (11.9)
District of residence	
Tanta	437 (32.0)
El Mehalla	355 (26.0)
Kafr El Zayat	118 (8.7)
Zefta	98 (7.2)
Samanoud	82 (6.0)
El Santa	103 (7.6)
Kotour	78 (5.7)
Basyoon	93 (6.8)

Table 2 Age and clinical characteristics of study population in Gharbiah, Egypt (1999-2007)	
Variable	*n* (%)
Age (yr)	
<40	300 (22.0)
≥40	1064 (78.0)
Basis of diagnosis	
Histology of primary	1210 (88.7)
Histology of metastases	58 (4.3)
Death certificate only	52 (3.8)
Others[1]	44 (3.2)
Tumor site	
Colon	856 (62.8)
Right	347 (40.5)
Left	331 (38.7)
NOS	178 (20.8)
Rectum	508 (37.2)
Grade	
I	96 (7.0)
II	702 (51.5)
III	155 (11.4)
IV	61 (4.5)
Unknown	350 (25.7)
Polyps	
Present	38 (2.8)
< 40 yr	9 (23.7)
≥ 40 yr	29 (76.3)
Male	19 (50.0)
Female	19 (50.0)
Not present	1326 (97.2)
Mucinous carcinoma	
Present	318 (23.3)
< 40 yr	102 (32.1)
≥ 40 yr	216 (67.9)
Not present	1046 (76.7)
Histopathology	
Adenocarcinoma	1186 (87.0)
Carcinomas[2]	50 (3.7)
Other specified types of cancers	16 (1.2)
Unspecified types of cancers	112 (8.2)

[1]Others include clinical only (2 cases), clinical/ultrasound/X-Ray (29 cases), exploratory surgery/autopsy (5 cases), specific biochemistry/immunology. test (1 case), cytology/hematology (5 cases), and unknown (2 cases); [2]carcinomas include squamous cell (7 cases) and other carcinomas (43 cases). NOS: Not otherwise specified.

Slightly more than half the cases were males (54%), giving a male:female ratio of 1.2:1. Patients designated as residing in an urban area constituted 55.1% of all patients. In any given year, between 135 -171 new cases of colorectal cancer were included in the registry. The Tanta district of the Gharbiah province had the most cases (32.0%) with the Kotour district seeing the least (5.7%).

The clinical and histopathologic characteristics of the colorectal cancer patients included in the study are illustrated in Table 2. Age and tumor site distribution of cases showed that 22.0% of all cases were under the age of 40 and 62.8% of cases had primary lesions in the colon with 37.2% having primary lesions in the rectum. The majority of tumors were grade II moderately-differentiated tumors (51.5% of cases) followed by 11.4% grade III. About 25.7% cases had no grade information. The vast majority of patients did not present with polyps (97.2%) though of the 38 patients who had polyps, 76.3% were over the age of 40. Mucinous carcinomas were present in only 23.3% of cases and over two-thirds of those patients (67.9%) were patients over the age of 40 years. Adenocarcinoma was the most common histopathologic type of tumors (87.0%).

Table 3 shows colorectal cancer incidence rates and incidence rate ratios by gender and urban-rural status. For both men and women, living in an urban area significantly increased the risk of developing colorectal cancer (either in the colon or the rectum). The same trend was present looking only at primary lesions in the colon and less pronounced for rectal cancers. Older urban men were more likely to develop rectal cancer while young urban men were more likely to develop colon cancer.

The comparison of the incidence rates and incidence rate ratios of colorectal cancer across the 8 districts are shown in Table 4 for patients under the age of 40 years and those 40 years and older. Compared to the low rates of the Zefta district, living in Tanta and Basyoon districts was significantly associated with increased risk of developing colorectal cancer for both young and old subjects. Living in El Mehalla and Kafr El Zayat was associated with significantly increased risk of developing colorectal cancer for subjects over 40 years only.

Compared to the low rates of colon or rectal cancers in the Zefta district, living in Tanta and Basyoon districts was associated with significantly increased risk of developing both primary colon and rectal cancers. Living in El Mehalla and Kafr El Zayat districts was significantly associated with increased risk of developing colon but not rectal cancer (Table 4).

Table 5 compares age standardized incidence rates by

Table 3 Incidence rates (per 10^5) and incidence rate ratios (95% confidence interval) by gender and urban-rural status for colorectal cancer patients in Gharbiah (1999-2007)

Age (yr)	Urban incidence		Rural incidence		Urban-rural IRR (95% CI)	
	Male	Female	Male	Female	Male	Female
Total						
5-9	0.17	0.00	0.00	0.00	-	-
10-14	0.00	0.35	0.00	0.16	-	2.23 (0.31, 15.82)
15-19	1.00	0.17	0.78	0.52	1.27 (0.47, 3.45)	0.32 (0.04, 2.59)
20-24	2.70	2.29	0.77	0.77	3.50 (1.57, 7.80)	2.97 (1.25, 7.05)
25-29	3.44	2.27	1.29	1.22	2.67 (1.30, 5.47)	1.86 (0.80, 4.30)
30-34	4.47	4.39	3.40	1.16	1.31 (0.74, 2.34)	3.79 (1.74, 8.28)
35-39	6.56	4.55	3.54	3.81	1.85 (1.07, 3.20)	1.19 (0.64, 2.24)
40-44	12.03	17.22	5.60	3.86	2.15 (1.35, 3.42)	4.46 (2.70, 7.35)
45-49	23.67	16.61	7.11	4.47	3.33 (2.22, 4.99)	3.72 (2.24, 6.16)
50-54	33.30	30.36	11.92	10.10	2.79 (1.93, 4.05)	3.00 (2.05, 4.40)
55-59	32.06	24.93	7.65	9.28	4.19 (2.66, 6.60)	2.69 (1.76, 4.11)
60-64	41.86	29.89	13.24	11.55	3.16 (2.03, 4.92)	2.59 (1.60, 4.18)
65-69	49.14	24.40	12.27	9.04	4.01 (2.34, 6.86)	2.70 (1.47, 4.95)
70-74	45.38	39.93	16.36	7.79	2.77 (1.43, 5.39)	5.13 (2.44, 10.77)
75+	40.95	36.28	16.36	11.99	2.50 (1.21, 5.19)	3.03 (1.52, 6.03)
Colon						
5-9	0.17	0.00	0.00	0.00	-	-
10-14	0.00	0.17	0.00	0.16	-	1.11 (0.10, 12.29)
15-19	0.33	0.17	0.50	0.52	0.67 (0.14, 3.21)	0.32 (0.04, 2.59)
20-24	1.80	2.10	0.39	0.43	4.67 (1.60, 13.67)	4.90 (1.70, 14.11)
25-29	1.72	1.59	0.55	0.51	3.12 (1.08, 8.98)	3.12 (0.99, 9.83)
30-34	2.98	2.32	1.70	0.58	1.75 (0.83, 3.70)	4.01 (1.34, 11.97)
35-39	4.56	2.42	2.32	2.45	1.97 (1.01, 3.83)	0.99 (0.43, 2.28)
40-44	7.78	8.61	3.63	2.18	2.14 (1.20, 3.82)	3.94 (2.00, 7.79)
45-49	14.95	10.79	5.69	2.61	2.63 (1.63, 4.23)	4.14 (2.16, 7.93)
50-54	22.38	15.67	7.48	6.59	2.99 (1.89, 4.75)	2.38 (1.44, 3.91)
55-59	22.81	16.97	5.54	5.95	4.12 (2.41, 7.03)	2.85 (1.69, 4.81)
60-64	28.21	21.59	6.62	5.96	4.26 (2.36, 7.70)	3.62 (1.94, 6.75)
65-69	27.30	14.85	6.43	5.24	4.25 (2.04, 8.86)	2.84 (1.29, 6.25)
70-74	26.27	26.04	12.27	4.67	2.14 (0.95, 4.85)	5.57 (2.16, 14.36)
75+	30.03	32.46	10.52	6.85	2.86 (1.18, 6.89)	4.74 (2.04, 10.98)
Rectum						
5-9	0.00	0.00	0.00	0.00	-	-
10-14	0.00	0.17	0.00	0.00	-	-
15-19	0.66	0.00	0.28	0.00	2.34 (0.58, 9.34)	-
20-24	0.90	0.19	0.39	0.34	2.34 (0.68, 8.07)	0.56 (0.06, 4.99)
25-29	1.72	0.68	0.74	0.71	2.34 (0.88, 6.22)	0.96 (0.25, 3.69)
30-34	1.49	2.06	1.70	0.58	0.88 (0.34, 2.24)	3.57 (1.17, 10.90)
35-39	2.00	2.12	1.22	1.36	1.64 (0.62, 4.30)	1.56 (0.59, 4.10)
40-44	4.25	8.61	1.97	1.68	2.16 (0.98, 4.73)	5.13 (2.44, 10.77)
45-49	8.72	5.81	1.42	1.86	6.13 (2.72, 13.85)	3.12 (1.39, 7.03)
50-54	10.92	14.69	4.44	3.51	2.46 (1.31, 4.61)	4.18 (2.28, 7.67)
55-59	9.25	7.96	2.11	3.33	4.38 (1.86, 10.33)	2.39 (1.15, 4.95)
60-64	13.65	8.30	6.62	5.59	2.06 (1.03, 4.13)	1.49 (0.67, 3.31)
65-69	21.84	9.55	5.84	3.81	3.74 (1.70, 8.24)	2.51 (0.97, 6.50)
70-74	19.11	13.89	4.09	3.12	4.67 (1.41, 15.52)	4.46 (1.34, 14.80)
75+	10.92	3.82	5.84	5.14	1.87 (0.50, 6.96)	0.74 (0.15, 3.68)

IRRs: Incidence rate ratios; 95% CI: 95% confidence itervals.

gender of the Gharbiah registry and the United States SEER data. Incidence rate of colorectal cancer was $5.5/10^5$ in Gharbiah ($6.1/10^5$ for males, $4.9/10^5$ for females). These rates were significantly lower than the colorectal cancer incidence rates seen in the United States of $32.0/10^5$ ($37.7/10^5$ for males, $27.4/10^5$ for females). While incidence rate of colorectal cancer for those under age 40 years in Gharbiah was slightly higher than the United States incidence rate for the same age group, the incidence rates for subjects 40 years and older in the United States were significantly higher than the corresponding rates for the same age groups in Egypt.

DISCUSSION

Analysis of the 1364 cases of colorectal cancer collected at the Gharbiah population-based cancer registry from 1999-2007 revealed the following important findings: First, a relatively high incidence of colorectal cancer in young subjects under age 40 years and significantly low

Table 4 Comparison of incidence rates for colorectal cancer patients in the 8 districts of Gharbiah (1999-2007)

District	Young (< 40 yr)		Old (≥ 40 yr)		Colon		Rectum	
	IR	IRR (95% CI)	IR	IRR (95% CI)	IR	IRR (95% CI)	IR	IRR (95% CI)
Tanta	1.52	1.93 (1.23, 3.04)	18.53	2.08 (1.62, 2.67)	3.35	2.25 (1.69, 3.00)	1.98	1.77 (1.26, 2.48)
El Mehalla	0.89	1.13 (0.70, 1.82)	14.85	1.66 (1.29, 2.14)	2.66	1.78 (1.33, 2.38)	1.36	1.21 (0.85, 1.72)
Kafr El Zayat	1.06	1.35 (0.77, 2.35)	12.44	1.39 (1.03, 1.89)	2.17	1.46 (1.03, 2.07)	1.44	1.29 (0.85, 1.95)
Samanoud	0.86	1.09 (0.58, 2.04)	11.40	1.28 (0.92, 1.78)	2.12	1.42 (0.98, 2.07)	1.10	0.98 (0.61, 1.58)
El Santa	0.93	1.18 (0.66, 2.11)	11.28	1.26 (0.92, 1.73)	1.99	1.33 (0.93, 1.91)	1.26	1.13 (0.73, 1.74)
Kotour	1.30	1.65 (0.93, 2.90)	9.56	1.07 (0.75, 1.52)	2.06	1.38 (0.94, 2.02)	1.09	0.97 (0.60, 1.58)
Basyoon	1.63	2.06 (1.18, 3.60)	13.81	1.55 (1.11, 2.15)	2.39	1.60 (1.10, 2.34)	1.97	1.76 (1.15, 2.70)
Zefta	0.79	1.00	8.93	1.00	1.49	1.00	1.12	1.00

IRR: Incidence rate ratio; IR: Incidence rate; 95% CI: 95% confidence interval.

incidence in subjects 40 years and older. Second, high proportion of tumors located in the rectum. Third, a vast majority of tumors (over 97%) did not have polyps. Fourth, living in an urban area was associated with higher rates of colorectal cancer, with variability in rates across the region.

Similar low rates of colorectal cancer in this population in Egypt ($6.9/10^5$ for males and $5.1/10^5$ for females) were reported by the MECC for the short period of 1999-2001[11]. The low rates were also reported from the Gharbiah cancer registry for the period of 2000-2002, where the age-standardized incidence rates for colorectal cancer was $6.5/10^5$ for males and $4.2/10^5$ for females[12]. The relatively high rate in subjects under age 40 years was reported by the MECC report in which Egypt had the highest incidence for both genders combined ($1.4/10^5$) and for males ($1.7/10^5$) than the rate in the same age group among Israeli Jewish and Arab populations, Jordanians and Cyprians[13]. High proportion of young-onset colorectal cancer was also reported in our previous hospital-based studies[5-7,14] that showed about 1/3 of all Egyptian colorectal cancer patients under age 40 years. It is unclear if the high young-onset rate is due to adoption of a more "westernized" lifestyle and diet, particularly in the younger generation[6,15] or due to intense environmental exposures with more susceptibility among the younger generations[15]. While our previous studies showed no familial aggregation among young patients to suggest HNPCC or similar syndromes[7,16,17], more recent studies of possible mismatch repair gene defects[18-21] or autosomal recessive inheritance[22] are warranted in this population, especially in absence of a strong family history and lack of distinct molecular characteristics among young-colorectal cancer patients in Egypt[7,17].

The high proportion of cancers that are located in the rectum in this study (37.2%) and the low ratio of colon/rectum tumors are characteristic of colorectal cancer in developing countries[23]. The high proportion of rectal cancer was reported in our previous hospital based studies[5-7]. However, the proportion of rectal cancer declined from about 50% to 37% perhaps due to the more accurate nature of the population-based studies or the changing life-style with westernization leading to higher proportions of colon than rectal cancers[23]. How-

Table 5 Age standardized incidence rates by gender in Gharbiah, Egypt and the United States Surveillance Epidemiology and End Results Program

	Gharbiah, Egypt 1999-2007			United States SEER 1999-2001			P value
	Total	Male	Female	Total	Male	Female	
Total cases	1364	737	627	55 480	27 892	27 588	
Total rate	5.5	6.1	4.9	32.0	37.7	27.4	< 0.0001[1]
< 40 yr	1.3	1.4	1.2	1.2	1.3	1.2	
40-59 yr	12.0	12.8	11.2	37.9	43.3	32.8	
60-69 yr	19.4	22.5	15.8	154.0	185.4	126.4	
> 70 yr	21.2	24.4	18.6	311.3	369.8	270.8	

[1]χ^2 test of total age standardized incidence rates, by age group, Gharbiah, Egypt 1999-2007 *vs* United States Surveillance Epidemiology and End Results Program (SEER) 1999-2001.

ever, the proportion of primary rectal colorectal cancers in this study is still high compared to Western countries, where only 27.9% of American colorectal cancers are primary rectum[13]. It is worth noting that recent studies in the United States showed increasing incidence of both young-onset colorectal cancer as well as the proportions of rectal cancers[24].

The very low rate of polyps reported in this study is unique. The low rate of polyps was also reported in our previous hospital-based studies[6,7]. It is important to note that pathologists in this population in Egypt report very few polyps in other segments of the resected colon not only in the cancer site. Causes of the very low rate of polyps may be related to diet rich in high fiber, legumes, and green vegetables[25,26] which is common in this population[27,28]. Other causes of the low polyp rate may be related to the intake of aspirin or aspirin- like compounds[29-32] which is also common in this population because of self-medication[33,34] or other molecular pathways that do not include polyps in the colorectal carcinogenesis in this population.

There are a number of distinctive environmental and possibly genetic factors that may contribute to the variable rates of urban/rural incidence in this population. Intense exposure to pesticides in this predominately agricultural region[8], industrial pollution[15,35-37], and high rate of consanguinity and first cousins' marriage[16,17] may also

lead to this variable cancer incidence and urban/rural rate risk differences.

The higher incidence rate of colorectal cancer in regions of the province may suggest differences in environmental exposures and/or variable access to medical care for colorectal screening or diagnosis. As there is a fairly reliable access to medical care for diagnosis and no screening facilities are available in the province of the registry[38], differences in risk factors across the region is most likely the cause of the variation in incidence rates. The main occupation in the Gharbiah province is agriculture. Further, pesticide manufacturing in Kafr El Zayat City and textile production in El Mehalla City are also important industries in the province.

This study had the following strengths: (1) the population-based data from the Gharbiah population-based cancer registry and the large sample size give an accurate picture of the state of colorectal cancer in Egypt; (2) the inclusion of key demographic and clinical data allowed for characterization of the clinical profile and suggestions of possible risk factors for colorectal cancer in this population; and (3) previous studies in the same population using hospital-based data were comparable to results from the population-based cancer registry. Weaknesses included inherent nature of population-based cancer registries of limited information on potential risk factors for colorectal cancer, such as diet and lifestyle habits, pesticide exposure, and family history because of lack of interviewing of patients.

In summary this study showed a relatively high incidence of colorectal cancer under age 40 years and a significantly low incidence in the age group of 40 years and older in this population in Egypt. The high proportion of rectal cancers and the vast majority of tumors without polyps are also interesting findings of the study. Regional differences in disease incidence in colon and rectal cancers in the region may reflect different etiologic patterns in this population. Future analytical studies should focus on further understanding of the etiology and pathogenesis of the disease in this population with extensive environmental exposures and possible genetic factors that may modulate the disease risk.

COMMENTS

Background
Previous hospital-based studies in Egypt showed low incidence of colorectal cancer and high proportion of young-onset disease. Egypt has a new reliable resource of a population-based registry in the Gharbiah region of the Nile Delta. The registry data for the period of 1999-2007 was used to examine epidemiologic, clinical and incidence rates of colorectal cancer in this population and to compare that with the results of the United States Surveillance Epidemiology and End Results Program (SEER).

Research frontiers
The vast majority of tumors (97.2%) had no polyps and 37.2% of the patients presented with primary lesions in the rectum. Colorectal cancer was more common in patients from urban (55%) than rural (45%) areas. Regional differences in colon and rectal cancer incidence in the 8 districts of the study province may reflect different etiologic patterns in this population. The registry data of Egypt shows a slightly higher incidence of colorectal cancer than the United States in subjects under age 40 years. The results also shows significantly lower inci-

dence of colorectal cancer in subjects over age 40 years compared to the same age group in the United States SEER.

Innovations and breakthroughs
This study confirms that patients over age 40 years in Egypt have significantly lower incidence of colorectal cancer than subjects in the same age group in the United States. This is the first study on a population-based scale to show the limited proportions of polyps in colorectal cancer patients in Egypt.

Applications
Future clinical and epidemiologic studies should investigate the etiologic factors related to the regional differences of colorectal cancer in this population in Egypt. Studies should also explore clinical and molecular pathways for the district age and polyp distribution of colorectal cancer.

Peer review
The quality of the data set is very important, especially in the population-based cancer registry.

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BRIEF ARTICLE

Faecal pyruvate kinase isoenzyme type M2 for colorectal cancer screening: A meta-analysis

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Abstract

AIM: To present a critical discussion of the efficacy of the faecal pyruvate kinase isoenzyme type M2 (faecal M2-PK) test for colorectal cancer (CRC) screening based on the currently available studies.

METHODS: A literature search in PubMed and Embase was conducted using the following search terms: fecal Tumor M2-PK, faecal Tumour M2-PK, fecal M2-PK, faecal M2-PK, fecal pyruvate kinase, faecal pyruvate kinase, pyruvate kinase stool and M2-PK stool.

RESULTS: Stool samples from 704 patients with CRC and from 11 412 healthy subjects have been investigated for faecal M2-PK concentrations in seventeen independent studies. The mean faecal M2-PK sensitivity was 80.3%; the specificity was 95.2%. Four studies compared faecal M2-PK head-to-head with guaiac-based faecal occult blood test (gFOBT). Faecal M2-PK demonstrated a sensitivity of 81.1%, whereas the gFOBT detected only 36.9% of the CRCs. Eight independent studies investigated the sensitivity of faecal M2-PK for adenoma (*n* = 554), with the following sensitivities: adenoma < 1 cm in diameter: 25%; adenoma > 1 cm: 44%; adenoma of unspecified diameter: 51%. In a direct comparison with gFOBT of adenoma > 1 cm in diameter, 47% tested positive with the faecal M2-PK test, whereas the gFOBT detected only 27%.

CONCLUSION: We recommend faecal M2-PK as a routine test for CRC screening. Faecal M2-PK closes a gap in clinical practice because it detects bleeding and non-bleeding tumors and adenoma with high sensitivity and specificity.

INTRODUCTION

Colorectal cancer (CRC) is the most frequent malignant disease in Europe according to an estimation of cancer incidence and mortality by the International Agency for Research on Cancer in Lyon, France[1]. In 2008, 436 000

persons were diagnosed with CRC, followed by breast cancer with 421 000 cases, lung cancer with 391 000 cases and prostate cancer with 382 000 cases. Approximately 212 000 patients died due to CRC that year, which makes it the second most common death from cancer (after lung cancer with approximately 342 000 deaths in 2008)[1]. Worldwide, in the developed countries about 1.167 million new cases of CRC and about 603 000 deaths due to CRC were estimated for 2007[2].

However, due to the long process of carcinogenesis in CRC (adenoma-carcinoma sequence), CRC has an overall good prognosis when diagnosed at an early stage. For that reason different CRC screening programs have been developed and are offered in various European countries.

The gold standard for early detection of colorectal neoplasia is colonoscopy. A great advantage of colonoscopy is that adenomas, the potential precursors of carcinogenesis, can be simultaneously detected and removed. However, the acceptance of screening colonoscopy among patients is low. For example, in Germany only 2.7% of insured people exercise their right to a colonoscopy even though it is reimbursed for people over 55 years old[3]. The most common *in-vitro* diagnostic method for CRC screening is the detection of occult blood in the stool using the guaiac-based faecal occult blood test (gFOBT). This test is based on the peroxidase activity of haemoglobin, which induces an oxidation and blue colouration of guaiac in the presence of hydrogen peroxide. Red meat and a number of vegetables may result in false positive results whereas vitamin C may result in false negative staining. Therefore, dietary restriction is recommended for three days prior to and during testing. A widespread criticism of gFOBT is its low sensitivity for adenomas and carcinomas (13%-50%)[4-8]. The immunological faecal occult blood tests (iFOBTs) specifically quantify human haemoglobin with antibodies. Comparative evaluations of immunochemical faecal occult blood tests from different manufacturers have revealed great variations in their respective sensitivities for colorectal adenoma detection[9,10].

The faecal pyruvate kinase isoenzyme type M2 (faecal M2-PK) test recognises a key enzyme controlling the metabolism of cells with a high proliferation rate, such as tumour cells, and thereby detects specific alterations in intestinal cells, such as polyps and CRC, as well as high-risk patients with acute or chronic inflammatory bowel diseases (IBD) (i.e., ulcerative colitis, Crohn's disease).

M2-PK is a special isoenzyme of pyruvate kinase, a key enzyme within glycolysis which catalyzes the ATP-producing conversion of phosphoenolpyruvate (PEP) to pyruvate. Depending upon the metabolic functions of the tissues, different isoenzymes of pyruvate kinase are expressed. During tumour formation the tissue-specific isoenzymes disappear and the pyruvate kinase isoenzyme type M2 is expressed[11]. In contrast to all other pyruvate kinase isoenzymes (type L, M1 and R) which consist of four subunits, the M2 pyruvate kinase isoenzyme may occur in a highly active tetrameric form as well as in a dimeric form with low activity. The dimeric form is nearly inactive and favours the channelling of glucose carbons into synthetic processes, such as nucleic acid, amino acid and fatty acid synthesis. The tetrameric form is highly active and favours the energy-regenerating conversion of PEP to pyruvate and lactate (the Warburg effect). In tumour cells, M2-PK is mainly found to be in the dimeric form and has therefore been termed "Tumour M2-PK". The dimerisation of M2-PK is induced by interaction with different oncoproteins, including pp60v-src-kinase, oncogenic fibroblast growth factor1 and human papilloma virus 16 E7[11].

The dimeric form of M2-PK is released from tumours into the blood and can be quantified by a sandwich enzyme-linked immunosorbent assay (ELISA; ScheBo Biotech AG, Giessen, Germany). About 40 studies have been published on M2-PK concentrations in blood since 1997. These demonstrate a significant increase in M2-PK and correlation with staging for the following tumours: melanoma, thyroid, breast, lung, kidney, oesophageal, gastric, pancreatic, colorectal, ovarian, cervical and renal cell cancer[12-19]. The long-term determination of M2-PK in EDTA-plasma is used as a tool for follow-up studies to monitor failure, relapse or success during therapy. In CRC and adenoma M2-PK is also released into the patients' faeces. A sandwich ELISA and a lateral flow rapid test (for doctor's office, point-of-care and laboratory use), both based upon two monoclonal antibodies which specifically recognise the dimeric form of M2-PK, are commercially available for the quantification of M2-PK in stool. The potential of the faecal M2-PK test for CRC screening has been evaluated in at least 17 different independent international studies. The objectives of this review were to obtain an overview of the currently available studies with faecal M2-PK and to present a critical discussion of the efficacy of the faecal M2-PK test for CRC screening.

MATERIALS AND METHODS

Search procedure for studies
In order to find the most relevant studies about faecal M2-PK and CRC screening, a literature search in PubMed and Embase was conducted using the following search terms: fecal tumor M2-PK, faecal tumour M2-PK, fecal M2-PK, faecal M2-PK, fecal pyruvate kinase, faecal pyruvate kinase, pyruvate kinase stool, M2-PK stool. In June 2011 this search revealed 34 publications dealing with faecal M2-PK[7,8,10,18,20-49] (Table 1). The ScheBo faecal M2-PK test was used in 33 publications, whereas one publication used another antibody combination and was therefore excluded. The following were also omitted from the meta-analysis: seven publications which summarized results from previous papers as reviews; three author-replies to questions about an existing published paper; one publication written in Bulgarian; two publications which investigated neither sensitivity nor specificity; seven publications that only referred to IBD (which was outside the scope of our review) (Table 1). The remaining 13 publications were included in the meta-analysis[7,8,10,30,31,33,35,37,41,44-46,49]. In

Table 1 Results of the literature search

Results	Reference
All papers dealing with faecal M2-PK found in a literature search of Pubmed and Embase	[7, 8, 10,18, 20-49]
Additional published studies known to the authors	[50-53]
Excluded papers - reasons for exclusion	
Unique combination of antibodies	[47]
Reviews	[18, 24, 26, 28, 34, 38, 42]
Author replies or comments	[27, 32, 40]
Paper in Bulgarian language	[29]
No sensitivities or specificities calculated	[21, 48]
Studies referred to IBD	[20, 22, 23, 25, 36, 39, 43]
Included papers	
Studies found in Pubmed and Embase	[7, 8, 10, 30, 31, 33, 35, 37, 41, 44-46, 49]
Published studies known to the authors	[50-53]

IBD: Inflammatory bowel diseases; **faecal M2-PK: Faecal pyruvate kinase** isoenzyme type M2.

Table 2 Overview of included studies

Reference	Country of study	Conflict of interest regarding faecal M2-PK
Shastri *et al*[7], 2006	Germany	None declared
Koss *et al*[8], 2008	United Kingdom	None declared
Möslein *et al*[10], 2010	Germany	None declared
Haug *et al*[30], 2008	Germany	None declared
Shastri *et al*[31], 2008	Germany	Coauthor Stein: Conference speaker for ScheBo Biotech AG
Haug *et al*[33], 2007	Germany	None declared
Mulder *et al*[35], 2007	The Netherlands	None declared
Ewald *et al*[37], 2007	Germany	None declared
Tonus *et al*[41], 2006	Germany	Non declared
Vogel *et al*[44], 2005	Germany	Tests performed by ScheBo Biotech AG
Naumann *et al*[45], 2004	Germany	None declared
Hardt *et al*[46], 2004	Germany	None declared
Tonus *et al*[49], 2009	Germany	None declared
Kloer *et al*[50], 2005	Germany	None declared
McLoughlin *et al*[51], 2005	Ireland	None declared
Bellutti *et al*[52], 2005	Germany	None declared
Schmidt *et al*[53], 2009	Germany	None declared

Faecal M2-PK: Faecal pyruvate kinase isoenzyme type M2.

addition, three posters from conferences[50-52] and a German doctoral thesis[53] known to the authors have been added to the list of relevant studies (Table 1). Hence, 17 published studies in total have been incorporated into the meta-analysis (Tables 1 and 2). For our meta-analysis the sensitivities for CRC and adenoma, positivity rates, as well as the specificities published within the individual papers were summarized in individual tables, together with the number of cases which underlie the calculated sensitivities and specificities. mean ± SD was calculated for the sensitivities and specificities of the combined data from the different studies using the Statistics package of SigmaPlot Version 11.0. The sensitivities for CRC and adenoma in all studies are based upon colonoscopy results.

Table 3 Published sensitivities of the faecal pyruvate kinase isoenzyme type M2 test for colorectal cancer

Reference	n (%)
Hardt *et al*[46], 2004	60 (73)
Naumann *et al*[45], 2004	27 (85.2)
Kloer *et al*[50], 2005	147 (79.6)
McLoughlin *et al*[51], 2005	35 (97)
Vogel *et al*[44], 2005	22 (77)
Shastri *et al*[7], 2006	74 (81.1)
Tonus *et al*[41], 2006	54 (78)
Haug *et al*[33], 2007	65 (68)
Mulder *et al*[35], 2007	52 (85)
Koss *et al*[8], 2008	32 (81)
Shastri *et al*[31], 2008	55 (78.2)
Schmidt *et al*[53], 2009	81 (80.3)
Sum	704
mean ± SD	80.3 ± 7.1

n: Number of colorectal cancer samples; %: Sensitivity.
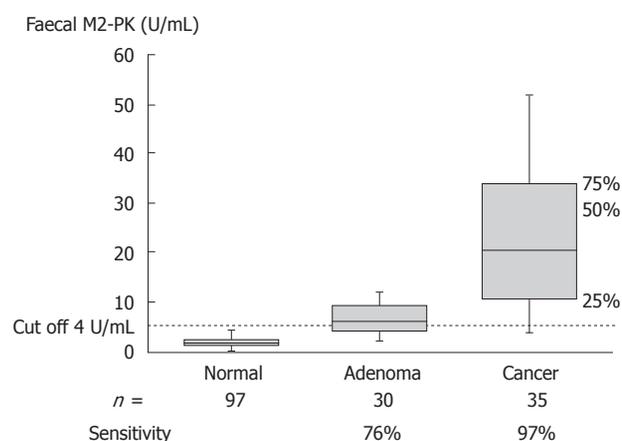
Figure 1 Faecal pyruvate kinase isoenzyme type M2 in healthy controls, patients with colorectal adenoma and colorectal cancer[51]. Faecal M2-PK: Faecal pyruvate kinase isoenzyme type M2.

Calculated specificities are either based on colonoscopy results or are authors' estimates derived from published prevalence data of CRC and adenoma in screening populations. In the absence of colonoscopies or estimated specificities, only the percentages of test-negative individuals were included in the tables.

Faecal M2-PK test
In all seventeen studies included in our meta-analysis, the M2-PK stool test from ScheBo Biotech AG in Giessen, Germany was used. This test is a sandwich ELISA based on two monoclonal antibodies which specifically recognise the dimeric form of M2-PK.

In accordance with the manufacturer's protocol all studies included a cut-off value of 4 U/mL. One study also included a lower cut-off value (3.33 U/mL[8]) and another also incorporated additional higher cut off values (5 U/mL and 6 U/mL[45]) to calculate the resultant sensitivities and specificities. To ensure comparability only those results obtained with the cut-off value of 4 U/mL are included in the meta-analysis.

Table 4 Correlation of faecal pyruvate kinase isoenzyme type M2 sensitivity with tumor node metastasis and Dukes classification *n* (%)

Reference	Tumor node metastasis classificatoin				Dukes classification			
	T1	T2	T3	T4	Dukes A	Dukes B	Dukes C	Dukes D
Kloer *et al*[50], 2005	9 (55.5)	18 (61.1)	49 (81.6)	12 (83.3)	23 (52.2)	24 (76.0)	26 (80.8)	17 (82.4)
Tonus *et al*[41], 2006	5 (60)	11 (64)	25 (89)	4 (100)	5 (60.0)	17 (76.0)	9 (89)	10 (90.0)
Haug *et al*[33], 2007	6 (67)	16 (44)	34 (71)	4 (100)	12 (67.0)	18 (61.0)	12 (67.0)	6 (100.0)
Schmidt *et al*[53], 2009	8 (57)	20 (84)	42 (79)	11 (91)				
Hardt *et al*[46], 2004	7 (57)	11 (64)	33 (78)	9 (78)				
Sum	35	76	183	40	40	59	47	33
mean ± SD	59 ± 5	63 ± 14	80 ± 7	90 ± 10	60 ± 7	71 ± 9	79 ± 11	91 ± 9

n: Number of samples tested; %: Sensitivity.

Table 5 Head-to-head comparison of the sensitivity for colorectal cancer of faecal pyruvate kinase isoenzyme type M2 and guaiac-based faecal occult blood test *n* (%)

Reference	CRC M2-PK	CRC gFOBT
Naumann *et al*[45], 2004	27 (85.2)	27 (62.9)
Vogel *et al*[44], 2005	22 (77)	22 (27)
Shastri *et al*[7], 2006	74 (81.1)	74 (36.5)
Koss *et al*[8], 2008	32 (81)	32 (21)
Sum	155	155
mean ± SD	81.1 ± 3.3	36.9 ± 18.5

n: Number of samples tested; %: Sensitivity; CRC: Colorectal cancer; gFOBT: Guaiac-based faecal occult blood test; M2-PK: Pyruvate kinase isoenzyme type M2.

Table 6 Sensitivity of faecal pyruvate kinase isoenzyme type M2 for adenoma *n* (%)

Reference	Adenoma without diameter	Adenoma < 1 cm ∅	Adenoma > 1 cm ∅
Naumann *et al*[45], 2004		11 (27.3)	13 (61.5)
McLoughlin *et al*[51], 2005	30 (76)		
Vogel *et al*[44], 2005	21 (48)		
Shastri *et al*[7], 2006		21 (28.6)	10 (20.0)
Mulder *et al*[35], 2007	47 (28)		
Koss *et al*[8], 2008		5 (20)	5 (60)
Shastri *et al*[31], 2008		48 (29.2)	21 (57.1)
Haug *et al*[30], 2008		254 (22.1)	68 (23.5)
Sum	98	339	117
mean ± SD	51 ± 24	25 ± 4	44 ± 21

n: Number of samples tested; %: Sensitivity; ∅: Diameter.

RESULTS

Sensitivity of faecal M2-PK for colorectal carcinoma

Sensitivity of the faecal M2-PK test for CRC was investigated and calculated in twelve independent studies (Table 3 and Figure 1), which found sensitivities of faecal M2-PK for detection of CRC between 68% and 97%. The mean sensitivity of all twelve studies is 80.3% ± 7.1%. These twelve studies measured faecal M2-PK concentrations in a total of 704 stool samples of patients with CRC, whereby 559 tested positive. Five studies considered the tumor node metastases and/or Dukes classification and showed a close correlation between the sensitivity of the faecal M2-PK test and staging (Table 4). The mean sensitivities ranged from 59% for T1 to 90% for T4 and from 60% for Dukes A to 91% for Dukes D. gFOBT studies from various countries showed much lower sensitivities for CRC which ranged between 13% and 50%[4-6]. The higher sensitivity of faecal M2-PK compared to gFOBT was confirmed in four studies which measured faecal M2-PK and gFOBT head-to-head in the same patients (Table 5). Combining all four studies, 155 samples from patients with CRC were tested for faecal M2-PK and gFOBT. M2-PK correctly detected 81.1% whereas the gFOBT detected only 36.9%.

Sensitivity of faecal M2-PK for adenoma

More than 90% of colorectal carcinomas evolve from adenoma *via* the adenoma-carcinoma sequence within 10 to 15 years. Therefore, the early detection and removal of adenoma is an important aspect in the prevention of CRC. The sensitivity of faecal M2-PK for adenoma was investigated in eight studies and ranged between 20% and 76%, whereby a clear dependency with the diameter of the adenoma is described (Table 6). In total, 339 adenomas with a diameter < 1 cm and 117 adenomas with a diameter > 1 cm were investigated. Twenty-five percent of the adenomas < 1 cm in diameter tested positive with the faecal M2-PK test and 44% of the adenomas > 1 cm were correctly detected. Three studies included a total of 98 stool samples from patients with adenoma of unclassified diameter. Faecal M2-PK concentrations above the cut-off were found in 51% of the samples. In direct comparisons of faecal M2-PK with gFOBT, 25% of patients with polyps < 1 cm tested positive with the M2-PK test whereas only 9% were identified by the gFOBT (Table 7). Fourty-seven percent of adenomas > 1 cm in diameter tested positive with the M2-PK test whereas the gFOBT detected only 27% (Table 7). One study with adenomas of unclassified diameter revealed a sensitivity of 48% for M2-PK in comparison to 9% for gFOBT. Möslein *et al*[10] combined adenomas > 1 cm in diameter and CRC to form a group with 55 cases of "advanced neoplasia". The resultant sensitivity of faecal M2-PK for advanced neoplasia was 27.3% whereas the sensitivity of gFOBT was only 9.1%. This study also included a head-to-head comparison of four iFOBTs from different manufacturers using the same 55 samples of patients with advanced neoplasia. With sensitivities of 7.3%, 8.5%, 18.9% and

Table 7 Head-to-head comparison of sensitivity for adenoma of faecal pyruvate kinase isoenzyme type M2 and guaiac-based faecal occult blood test *n* (%)

Reference	Adenoma < 1 cm ∅ M2-PK	Adenoma < 1 cm ∅ gFOBT	Adenoma > 1 cm ∅ M2-PK	Adenoma > 1 cm ∅ gFOBT	Adenoma w/o ∅ M2-PK	Adenoma w/o ∅ gFOBT
Naumann *et al*[45], 2004	11 (27.3)	11 (18.2)	13 (61.5)	13 (30.8)		
Vogel *et al*[44], 2005					21 (48)	21 (9)
Shastri *et al*[7], 2006	21 (28.6)	21 (9.5)	10 (20.0)	10 (30.0)		
Koss *et al*[8], 2008	5 (20.0)	5 (0.0)	5 (60.0)	5 (20.0)		
Sum	37	37	28	28	21	21
mean ± SD	25 ± 5	9 ± 9	47 ± 24	27 ± 6		

n: Number of samples tested; %: Sensitivity; w/o ∅: Without measurement of diameter; ∅: Diameter; gFOBT: Guaiac-based faecal occult blood test; M2-PK: Pyruvate kinase isoenzyme type M2.

Table 8 Measurements of faecal pyruvate kinase isoenzyme type M2 in stool samples of healthy individuals

Reference	No. of healthy participants	Test-negative participants (%)	Colonoscopy (yes/no)	Specificity (%)
Belluti *et al*[52], 2005	2787	91.6	No	97.4 (e)
McLoughlin *et al*[51], 2005	97	98	Yes	98
Tonus *et al*[41], 2006	42	93	Yes	93
Ewald *et al*[37], 2007	1906	90.4	No	
Haug *et al*[33], 2007	917	78.6	No	
Koss *et al*[8], 2008	13	100.0	Yes	100.0
Tonus *et al*[49], 2009	4854	91.2	No	93.4 (e)
Möslein *et al*[10], 2010	796	89.5	Yes	89.5
Sum	11 412			
mean ± SD		91.5 ± 6.4		95.2 ± 3.9

e: Estimated specificities calculated by authors based on the sensitivity of faecal pyruvate kinase isoenzyme type M2 for colorectal cancer (CRC) and advanced neoplasia, and the prevalence of CRC and advanced adenoma.

20%, respectively, all four iFOBTs were less sensitive than faecal M2-PK.

Specificity of faecal M2-PK for colorectal carcinoma

The specificity of an *in-vitro* diagnostic test reflects the proportion of correctly identified negatives. Consequently, the composition of the control group has a profound effect on the specificity. By its very definition, screening is used in a population to detect a disease in individuals without signs or symptoms of that disease. Therefore, symptoms in the gastrointestinal tract, such as pain, visible blood in the stool or known inflammation are not appropriate for inclusion into the control group of a CRC screening study. In total, seventeen publications calculated specificities for the M2-PK stool test. Nine of these studies included patients from hospitals (clinical settings instead of screening settings) with positive gFOBTs and with inflammation and/or other symptoms in the gastrointestinal tract into the control group and hence these studies have been discounted from our evaluation of the specificity of faecal M2-PK[7,30,31,35,44-46,50,53]. Eight studies, comprising 11 412 samples in total, had control groups which conformed to the correct composition for screening studies (Table 8, Figures 2 and 3). Ninty one point five percent tested negative which means that about 9% of those tested had a faecal M2-PK value above the cut-off value of 4 U/mL. Colonoscopies were performed in four studies[8,10,51,41] (Table 8) and revealed specificities of 98% (*n* = 97), 93% (*n* = 42), 100% (*n* = 13) and 89.5%

(*n* = 796). In study 49 with 4854 participants, the authors calculated an estimated specificity of 93.4% based on a prevalence of CRC of 2%. Based on a prevalence of 0.5% for CRC and 18% for advanced adenoma, the authors of study 52 with 2787 participants calculated an estimated specificity for colorectal neoplasia of 97.4%. The screening in study 49 with 4854 participants describes a continuous increase in the percentage of faecal M2-PK positive volunteers with age from 30 years old upwards (Figure 3).

DISCUSSION

With a sensitivity of about 80% for CRC and 44% for adenoma > 1 cm, faecal M2-PK outclasses the gFOBT which has sensitivity between 13% and 50% for CRC (Tables 3-7, and literature[4-6]). The superiority of faecal M2-PK may be due to the fact that M2-PK is a metabolic biomarker which is characteristic for the metabolic state of tumour cells and their precursors, whereas detection of bowel cancer using the gFOBT is restricted to bleeding tumours and adenoma. Therefore, faecal M2-PK has the advantage that it detects both bleeding as well as non-bleeding tumours and adenoma and will close a gap in clinical practice. Conversely, faecal M2-PK does not have false positive results due to various non-cancerous sources of bleeding, e.g., haemorrhoids and fissures. Screening studies involving a total of more than 11 000 healthy subjects have demonstrated a mean specificity of 95.2% for the detection of CRC/advanced neoplasia with faecal M2-
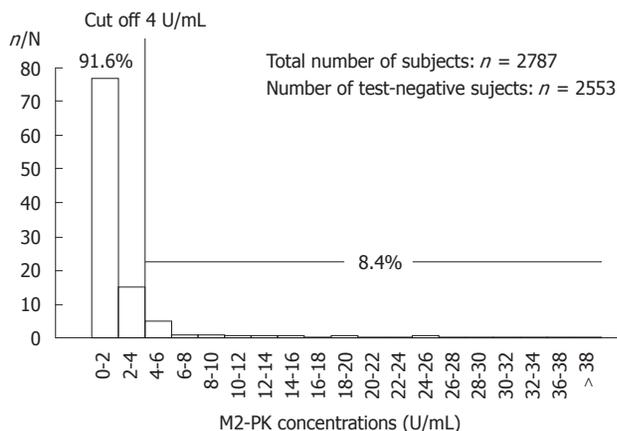
Figure 2 Distribution of faecal pyruvate kinase isoenzyme type M2 concentrations in a screening collective of 2787 participants aged from 45 to 65 years[52]. *n*: Number of test negative; N: Total number of subjects; M2-PK: Pyruvate kinase isoenzyme type M2.

Figure 3 Percentage of faecal pyruvate kinase isoenzyme type M2-positive volunteers by age group (from Tonus *et al*[49]).

PK. The specificities were 100%, 98%, 93% and 89.5%, respectively, in studies which incorporated colonoscopies; 97.4% and 93.4% in studies with estimated specificities; and 90.4% and 78.6 % in studies without colonoscopies (Table 8). This demonstrates that specificities were higher in studies with confirmatory colonoscopies in comparison to studies without colonoscopies. Whilst gFOBT specificities ≥ 94% are reported in the literature[5,6], the authors of a meta-analysis of over 440 000 subjects from six independent studies concluded that more than 80% of the positive gFOBT results are actually false positives[54]. In most studies the calculated specificities are based on the results of colonoscopy. Colonoscopy is the gold standard for early detection of CRC and polyps and has the advantage that polyps, the potential precursors of carcinogenesis, can be simultaneously detected and removed. However, recent studies have revealed that colonoscopies may have false negative results, e.g., due to suboptimal bowel preparation. For example, a systematic review which summarized six studies totaling 465 patients who had undergone two colonoscopies on the same day revealed a pooled miss rate of 22% for polyps of any size[55].

IBD may also be a cause of increased faecal M2-PK levels and hence detection of previously undiagnosed patients by faecal M2-PK is another advantage of the test, whereas those patients with known IBD are subject to their own endoscopic monitoring program and are not categorized as suitable for inclusion in a non-invasive CRC screening program.

The cost of one faecal M2-PK ELISA test is about 15-25 US$. In comparison, based on 2004 data from privately insured beneficiaries, costs were estimated to be about 557 US$ (range: 150-1112 US$) for a colonoscopy, 174 US$ (range: 54-392 US$) for a flexible sigmoidoscopy and 7 US$ (range: 2-16 US$) for a guaiac faecal occult blood test[56].

In conclusion, faecal M2-PK, either as an ELISA or as a lateral flow rapid test, is a cost-effective and easy-to-perform routine test. In contrast to the gFOBT, only one small stool sample (from a single stool passage), which may be collected with a convenient stool sample device, is necessary and no dietary restrictions are needed. Faecal M2-PK is an appropriately sensitive tool to pre-select those patients who require colonoscopy for further diagnostic confirmation or exclusion of CRC. Based on the current data we recommend the use of faecal M2-PK as a routine *in-vitro* diagnostic test for CRC screening.
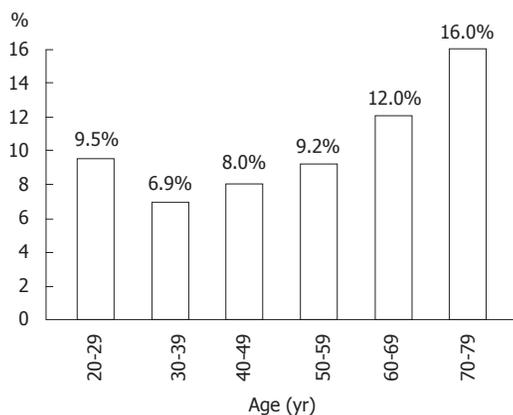
COMMENTS

Background
Colorectal cancer (CRC) is the most frequent malignant disease worldwide. The gold standard for early detection of colorectal neoplasia is colonoscopy. However, the acceptance of screening colonoscopy by potential screenees is low. Faecal pyruvate kinase isoenzyme type M2 (faecal M2-PK) is an *in-vitro* diagnostic test which recognizes a specific metabolic characteristic of proliferating cells in 4 mg stool samples. The simplicity of sample collection can encourage participation in CRC screening programs.

Research frontiers
The sensitivity and specificity of faecal M2-PK for CRC screening has been investigated in numerous publications. Here the paper presents a critical discussion of the efficacy of faecal M2-PK for CRC screening based on the accumulated data from currently available studies.

Innovations and breakthroughs
The most established in-vitro diagnostic test for CRC screening is the guaiac-based faecal occult blood test (gFOBT). In contrast to the FOBTs, faecal M2-PK detects bleeding and non-bleeding tumors. With a sensitivity of about 80% for CRC and 44% for adenoma > 1 cm, faecal M2-PK outclasses the gFOBT which has a sensitivity between 13% and 50% for CRC.

Applications
This meta-analysis summarizes the results of 17 published studies evaluating the faecal M2-PK test for CRC screening. The data will help to critically assess the efficiency of the faecal M2-PK test in comparison to other *in-vitro* diagnostic tests for CRC screening.

Peer review
This is a meta-analysis about screening CRC with fecal MK-pyruvate kinase.

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BRIEF ARTICLE

Clinical trial: *Lactobacillus plantarum* 299v (DSM 9843) improves symptoms of irritable bowel syndrome

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Abstract

AIM: To assess the symptomatic efficacy of *Lactobacillus plantarum* 299v (*L. plantarum* 299v) (DSM 9843) for the relief of abdominal symptoms in a large subset of irritable bowel syndrome (IBS) patients fulfilling the Rome III criteria.

METHODS: In this double blind, placebo-controlled, parallel-designed study, subjects were randomized to daily receive either one capsule of *L. plantarum* 299v (DSM 9843) or placebo for 4 wk. Frequency and intensity of abdominal pain, bloating and feeling of incomplete rectal emptying were assessed weekly on a visual analogue scale while stool frequency was calculated.

RESULTS: Two hundred and fourteen IBS patients were recruited. After 4 wk, both pain severity (0.68 + 0.53 *vs* 0.92 + 0.57, $P < 0.05$) and daily frequency (1.01 + 0.77 *vs* 1.71 + 0.93, $P < 0.05$) were lower with *L. plantarum* 299v (DSM 9843) than with placebo. Similar results were obtained for bloating. At week 4, 78.1 % of the patients scored the *L. plantarum* 299v (DSM 9843) symptomatic effect as excellent or good *vs* only 8.1 % for placebo ($P < 0.01$).

CONCLUSION: A 4-wk treatment with *L. plantarum* 299v (DSM 9843) provided effective symptom relief, particularly of abdominal pain and bloating, in IBS patients fulfilling the Rome III criteria.

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most frequent digestive tract disorders encountered by general practitioners and gastroenterologists. IBS is a functional bowel disorder characterized by chronic and relapsing abdominal pain or discomfort associated with altered bowel habits. The primary aim of any treatment is the relief of abdominal pain which can significantly impair the patient's quality of life. According to published guidelines, the main treatment options for abdominal pain include antispasmodics or anti-depressants at low dose while anti-

diarrheal or laxative drugs are given to improve transit disturbances[1,2]. However, in many cases, all these options remain disappointing for the relief of abdominal pain. The therapeutic efficacy in IBS is probably impacted by the heterogeneous pathogenesis of the disease which includes altered intestinal motility, visceral hypersensitivity, abnormal brain-gut interactions, food intolerance, altered intestinal permeability and post infectious and/or inflammatory changes[3].

Recently, the deleterious role of qualitative or quantitative alterations of gut microbiota at the onset of symptoms has been emphasized. Therefore, a rationale exists to discuss the therapeutic use of probiotics, which are live microorganisms conferring health benefits to the host when ingested in adequate amounts[4]. Clinical evidence regarding the efficacy of some probiotic strains to improve IBS symptoms has recently emerged[5,6], although the mechanism of action of probiotics on IBS symptoms is not completely understood. Some probiotics bind to small and large bowel epithelium and may produce substances with antibiotic properties, while others compete for attachment and thereby reduce invasion by pathogenic organisms[7]. Probiotics also modulate gastrointestinal luminal immunity by changing the cytokine and cellular milieu from a pro-inflammatory to anti-inflammatory state[8]. They may also convert undigested carbohydrates into short chain fatty acids, which act as nutrients for colonocytes and affect gut motility[4].

Lactobacillus plantarum 299v (*L. plantarum* 299v) (DSM 9843) is a probiotic strain able to reside in the human colonic mucosa *in vivo* due to a specific mechanism of mannose adhesion[7]. *L. plantarum* 299v (DSM 9843) also increases the amount of carboxylic acid, particularly acetic and propionic acids, in the stools of healthy volunteers[9]. The strain has shown antibacterial activity against several potential pathogenic agents such as *Listeria monocytogenes*, *Escherischia coli*, *Yersinia enterolytica*, *Enterobacter cloacae* and *Enterococcus faecalis*[10]. *L. plantarum* 299v (DSM 9843) also has beneficial immunomodulatory activity *via* an increased interleukin-10 synthesis and secretion in macrophages and T-cells derived from the inflamed colon. And recently, an experimental study reported that *L. plantarum* 299v (DSM 9843) increased the transcription and excretion of the mucins MUC2 and MUC3 in goblet cells[11,12].

Three single-centre studies have tested the clinical efficacy of *L. plantarum* 299v (DSM 9843) in IBS patients[13-15]. Two trials have demonstrated significant benefits in comparison with placebo on improvement of flatulence scores[13] and a reduction of abdominal pain[14] while the results of the third trial, based on only 12 patients, were not conclusive. The aim of the present randomized, double-blind, placebo controlled clinical trial was to assess the symptomatic efficacy of *L. plantarum* 299v (DSM 9843) in a larger subset of IBS patients fulfilling the Rome III criteria.

MATERIALS AND METHODS

Patients
Participants (*n* = 214) were recruited by general practitio-

ners in four clinical centres in India: one in Mumbai, two in Chennai and one in Bangalore. Subjects between 18-70 years of age with IBS according to the Rome III criteria were eligible for inclusion. All subjects had a colonic examination at baseline to exclude any organic disease while an intestinal infection was excluded by stool cultures in any patient in whom this diagnosis was suspected. Subjects with severe chronic medical disease including colorectal and other gastrointestinal diseases were excluded. Pregnant and breast-feeding women and patients with dietary habits which might interfere with the assessment of the study product or patients with known allergy to the study product components were also excluded. Throughout the study, the subjects were not allowed to consume any other probiotic and were encouraged not to change their usual dietary and physical exercise habits.

Study design
This study was designed as a multicentre double blind, placebo-controlled study with parallel groups to assess the beneficial effects of a daily consumption of *L. plantarum* 299v (DSM 9843) on IBS symptoms. Treatment duration was 4 wk with 3 follow-up visits at weekly intervals. The study protocol was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee. All volunteers gave written informed consent prior to participation in the study.

Study products
The test product was a probiotic preparation containing a mixture of freeze-dried lactic acid bacteria and excipients. The lactic acid bacteria strain was *L. plantarum* 299v (DSM 9843). It is deposited at the DSM collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under number DSM 9843. The test product contained 10 billion colony-forming units (cfu) per capsule in a potato starch and magnesium stearate base. The control product contained potato starch (97%) and magnesium stearate (3%). Both the test and control products had a similar appearance, texture and taste. Both products were specifically prepared for the study and provided by the Rosell-Lallemand Institute (Blagnac, France).

Assessments and study endpoints
The primary endpoint was the improvement of the frequency of abdominal pain episodes. Secondary endpoints were changes in severity of abdominal pain, changes in frequency and severity of abdominal bloating and in feeling of incomplete rectal emptying. Both frequency of abdominal pain and feeling of incomplete rectal emptying were assessed weekly using a four-point scale ranging from 1 (only occasional symptom) to 4 (daily symptom). Symptom severity (abdominal pain, abdominal bloating and feeling of incomplete rectal emptying) was rated on a visual analogue scale (VAS 1-10) and converted to a 4 point scale ranging from 0 (No pain , VAS = 0) to 3 (Severe, VAS = 8 to 10).

The daily number of stools and bloating episodes were calculated and registered at each visit. At the end

Table 1 Baseline characteristics of the subjects between the two groups (mean ± SD)

	L. plantarum 299v (DSM 9843) (*n* =108)	Placebo (*n* =106)	*P*
Age (yr)	36.53 ± 12.08	38.40 ± 13.13	NS
Men/women	70/38	81/25	NS
IBS duration (yr)	3.4	4.6	NS
Abdominal pain frequency	2.1 ±1.01	1.98±0.91	NS
Abdominal pain severity	1.24 ± 0.60	1.20 ± 0.63	NS
Bloating severity	1.07 ± 0.62	1.14 ±0.64	NS
Stool frequency	3.94 ± 1.51	3.69 ± 1.34	NS
Pure vegetarians (%)	30.5	20.2	NS
Daily yoghurt intake (%)	46.7	42.1	NS

No significant differences were found between the groups for all the variables tested. NS: No significant; *L. plantarum* 299v: *Lactobacillus plantarum* 299v.

Figure 1 Flow-chart of the study. *L. plantarum* 299v: *Lactobacillus plantarum* 299v; FAS: Full analysis set; ITT: Intention to treat.

of the 4-wk treatment period, both the patient's and the practitioner's opinion about the overall efficacy of the treatment were recorded using a 4-point scale, from "poor" to "excellent".

Regarding safety assessment, blood samples were taken at baseline and week 4 in each patient for the assessment of blood cell counts, glycaemia, blood urea nitrogen and liver function tests. Physical examinations and verification of any adverse events were performed at each visit.

Sample size and randomization

The sample-size calculation was based on the main outcome, the frequency of abdominal pain episodes. The trial sample size required to detect a significant difference of 20% between the two groups with an 80% power and 5% statistical significance level was calculated to be at least seventy-nine patients per group. Taking into account that all subjects who withdrew prematurely were not replaced, 214 subjects were randomised according to a computer-generated randomization list in the ratio 1:1. For each site, randomization charts were provided to investigators keeping a 1:1 ratio. All investigators, patients and monitors were blinded throughout the study. To ensure allocation concealment, packaging and labelling were performed by a third party, and the randomization code was kept in a secure place during the study.

Statistical analysis

All the analyses of efficacy were performed with full analysis set (FAS) population. The FAS population corresponds to all randomised subjects who took at least one dose of the study drug and who had at least one post-baseline efficacy assessment. Overall assessment of symptoms were analysed using a repeated-measures analysis of variance (ANOVA) with time, treatment group, interaction time x product and baseline score as fixed factors for each period.

RESULTS

The flow chart of the study is given in Figure 1. A total
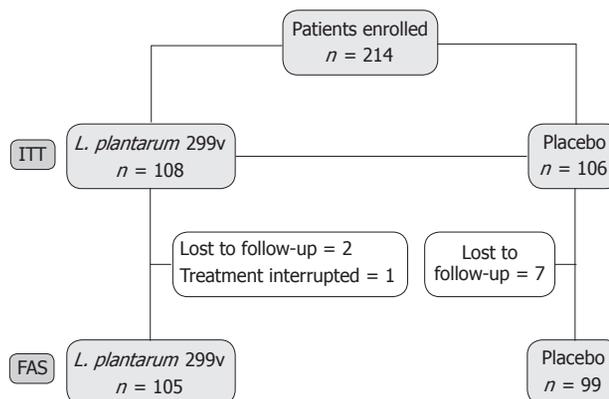
of 214 patients were randomized and 108 subjects assigned to receive *L. plantarum* 299v (DSM 9843) group and 106 patients the placebo. Among these 214 subjects, 10 were excluded, either because they did not complete the entire 4-wk double-blind period, or because they did not provide any available data about the treatment period. A majority of patients were IBS-D patients, 63.89% and 60.3% in *L. plantarum* 299v (DSM 9843) and placebo groups, respectively. Baseline characteristics of the two groups are given in Table 1.

Frequency of digestive symptoms

The mean changes over this 4-wk period of the frequency of each digestive symptom are shown in Figure 2. The decrease of abdominal pain frequency was significantly higher in the *L. plantarum* 299v (DSM 9843) group than in the placebo group at weeks 3 and 4. At the end of week 4 the mean frequency was reduced significantly by 51.9% in the *L. plantarum* 299v group in comparison with the 13.6% reduction in the placebo group. Overall reductions in stool frequency, bloating and feeling of incomplete emptying frequency were also significantly greater in the *L. plantarum* 299v (DSM 9843) group when compared with the placebo group over the 4-wk period (*P* < 0.05). The effects of both treatments on stool frequency are shown in Figure 3. A significant reduction of the daily number of stools was observed with *L. plantarum* 299v (DSM 9843) after the second week of treatment.

Severity of digestive symptoms

The change in mean severity of abdominal pain over the 4-wk period was analysed on the VAS. At the end of the 4th week, the mean score was reduced by 45.2% in the *L. plantarum* 299v (DSM 9843) group and reduced by only 23.3% in the placebo group (Figure 2A). The weekly analysis of this score showed significantly lower scores at weeks 2, 3 and 4 in the *L. plantarum* 299v (DSM 9843) group in comparison with placebo. The decrease of the mean scores of severity of abdominal bloating and feeling of incomplete emptying were also statistically higher in the *L. plantarum* 299v (DSM 9843) group when compared to the placebo group at weeks 3 and 4 (Figure 2B and C).
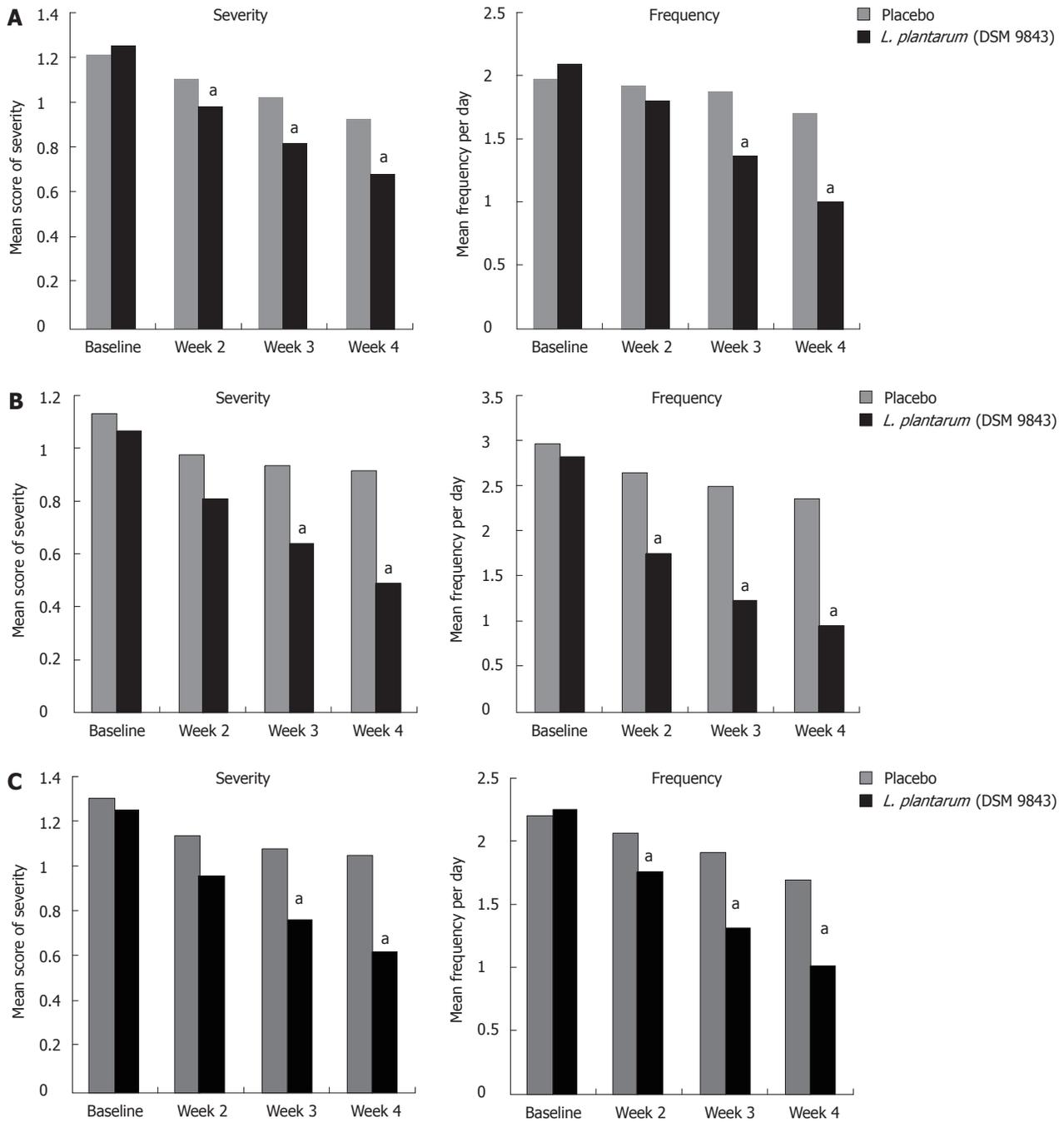
Figure 2 Changes in frequency and severity of symptoms in both groups. A: Abdominal pain; B: Bloating; C: Feeling of incomplete evacuation. *L. plantarum* (DSM 9843): *Lactobacillus plantarum* 299v. [a]*P* < 0.05 *vs* baseline group.

Overall assessment

The percentage of patients who considered the efficacy of the treatment they received as good or excellent was significantly higher in the *L. plantarum* 299v (DSM 9843) group than in the placebo group (78.1% *vs* 8.1%) (Figure 4). Similar results were observed when the efficacy was estimated by the investigators (82.8% *vs* 11.1%) (Figure 4).

Comparative efficacy according to dietary habits

Yoghurt consumption did not affect the results and did not induce any difference between the two arms of treatment (data not shown). The frequency of abdominal pain was also not different between the two arms when the vegetarian or non vegetarian status was considered. However, the severity of the abdominal pain with *L. plantarum* 299v (DSM 9843) was lower in the vegetarians than in the non-vegetarians at weeks 2, 3 and 4 (*P* < 0.05).

Safety

No significant side-effect was reported in any group during the 4 wk of treatment. The only adverse event reported was a transient vertigo onset by one of the patients who received *L. plantarum* 299v (DSM 9843). No change in blood parameters was detected throughout the study.
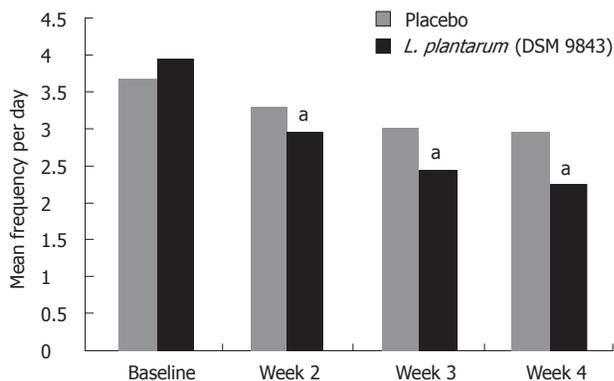
Figure 3 Changes in stool frequency in both groups. *L. plantarum* (DSM 9843): *Lactobacillus plantarum* 299v. [a]*P* < 0.05 *vs* baseline group.
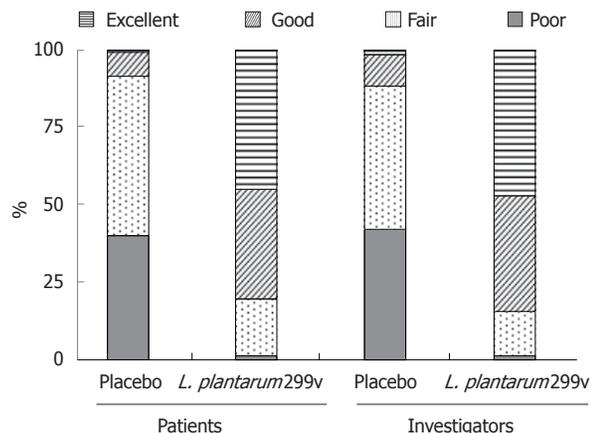
Figure 4 Overall assessment of the treatment efficacy by the patients and by the investigators.

DISCUSSION

The present placebo-controlled trial demonstrated that in an Indian population *L. plantarum* 299v (DSM 9843) is a probiotic strain able to relieve IBS symptoms, particularly abdominal pain and bloating, in IBS patients fulfilling the Rome III criteria. Abdominal pain was chosen as the primary end point because it is the major symptom leading to the seeking of medical advice by IBS patients. This trial was designed for a group of IBS patients of any subtype, complaining of moderate IBS symptoms and recruited by general practitioners. Several trials with probiotics have involved mainly IBS-D patients but microbiological studies have emphasized that qualitative changes of the microbiota exist in all IBS sub-types[16]. Therefore, we considered that any IBS patient, whatever the subtype, could be eligible to participate. In the present study, the majority of recruited participants were males as compared to previous trials where approximately two-thirds of study subjects were females. The female predominance in IBS patients reported in the West has not been observed in Asian populations, particularly in India. Two major recent community studies reported higher prevalence of IBS in the male population. In Mumbai, male prevalence was 7.9% *vs* female prevalence of 6.9%, and in a pan-Indian study male prevalence was 4.3% *vs* female prevalence of 4.0%[17]. However, other population surveys in the Indian subcontinent have reported an IBS prevalence of 8.5% using the Rome I criteria and demonstrated a female predominance similar to Western countries[18]. The notable gender difference between the population of this study and that of previously published trials can also be explained by the fact that, in the Indian subcontinent but not in other parts of Asia, men seem to have a greater access to healthcare[19]. However, data about the consultation behaviour of the community groups are not all in agreement. In the recent large survey conducted by the Indian Society of Gastroenterology Task Force (3000 IBS patients and 4500 community subjects in 18 centres), 33% of men and 38% of women had consulted a doctor in the preceding 12 mo[20]. Eating behaviours of the patients enrolled in this trial were also somewhat different from that of Western IBS patients

with a high percentage of pure vegetarians and with daily yoghurt consumption in almost half of the cases. Due to the possible interactions between nutrients and bacteria, we cannot exclude that this regimen might have influenced the therapeutic results even if eating behaviours were not different between the two groups. We have even observed that *L. plantarum* results on abdominal pain intensity were better in vegetarian than in non vegetarian IBS patients. This suggests that the symptomatic effect of the strain could be, at least partly, related to interactions between the luminal content and *L. plantarum* or that the strain affects the luminal metabolism of nutrients. However, the design of our study does not allow us to conclude that this is indeed the case.

This trial was performed according to the Rome III guidelines on design of trials for functional GI disorders[21] in order to demonstrate statistical superiority to placebo with a double-blind, placebo-controlled parallel design and outcome measures including both the effect of the treatment on the main symptom, i.e., abdominal pain, and a global assessment of the treatment efficacy to obtain adequate relief. Several clinical trials testing the symptomatic efficacy of probiotics in IBS were longer than this trial. However, a duration of treatment of 4 weeks follows not only the Rome III guidelines but is also the recommendation of international agencies[22]. One potential weakness of this study was the choice of a four-point Likert scale to analyze the frequency of symptoms instead of a score such as the IBS symptom severity scale that has been shown to be responsive to treatment effect[23].

We enrolled patients with moderate abdominal pain. Some studies have suggested that the achievement of a satisfactory relief end-point was significantly influenced by baseline symptom severity[23,24]. However, the concern that baseline severity compromises the achievement of an end point, such as satisfactory relief, does not appear to affect the current design of clinical trials. For example, trials with 5-HT3 antagonists[25] or antidepressant at low dose[26] or even with a non pharmacological approach[27] have not confirmed the impact of baseline severity on the

achievement of an adequate relief as a trial end point[17,28].

In accordance with previous findings in many trials, IBS patients who received placebo exhibited a significant improvement with time. However this improvement was lower than in the *L. plantarum* group and the overall number of patients in the placebo group who considered themselves as improved was low. Furthermore, in the present study, the placebo results were lower than that calculated in a recent meta-analysis of 73 randomized controlled trials (RCTs) reporting a pooled placebo response of 37.5%. But the same meta-analysis of the factors affecting placebo response rate outlined that rates were significantly higher in European RCTs[29]. The percentage of patients who considered the efficacy of the treatment as good or excellent was very high (78.1%) in the *L. plantarum* 299v (DSM 9843) group and low in the placebo group (8.1%). This result cannot be explained only by the greater effects of *L. plantarum* 299v (DSM 9843) *vs* placebo on each IBS symptom. This satisfaction rate could also be explained by a possible efficacy of the strain on upper abdominal symptoms that are very frequent in Indian IBS patients. Indeed, the Indian Society of Gastroenterology Task Force have outlined that 49% of Indian IBS patients reported epigastric pain, and that 70% complained of upper abdominal fullness or bloating rather than pain[20].

Three studies using *L. plantarum* 299v (DSM 9843) have been published prior to this trial. In the first study, Nobaek *et al*[13] enrolled 60 IBS patients and compared *L. plantarum* 299v to placebo to determine whether endogenous colonic flora could be altered by probiotic consumption. Multiple secondary symptom-based end-points were also evaluated. The active treatment period lasted 4 wk after a 2-wk observation period. Compared with placebo, a statistically significant decrease in flatulence was observed during the second half of the treatment period but only 52/60 patients were included in the analysis of this secondary endpoint. In another study, Niedzielin *et al*[14] enrolled 40 IBS patients and assessed abdominal pain and global IBS symptoms as primary and secondary outcomes, respectively. At 4 wk, 20/20 patients in the *L. plantarum* 299v group compared to 11/20 in the control group had complete resolution of their pain (P = 0.0012). Moreover, 19/20 patients in the *L. plantarum* 299v group compared to 3/20 patients in the control group also experienced improvement in their global IBS symptoms (P < 0.0001). In both trials, no adverse effects were identified. The final study, performed by Sen *et al*[15], showed no significant improvement but it was a pseudo-randomized study with only 12 patients with a cross-over design and evaluated changes of a composite score of IBS symptoms. At 8 wk, no significant differences were identified between groups[15]. Given the significant differences in the enrolled populations, study designs, outcome variables, and statistical analyses, it is difficult to make comparisons across the studies and all three previous studies suffered from multiple design flaws.

In conclusion, the present study shows the potential benefit of a particular strain *L. plantarum* 299v (DSM 9843), in the management of IBS. Further studies are warranted in order to identify the mechanism of the probiotic's potential beneficial effect.

COMMENTS

Background
Lactobacillus plantarum 299v (*L. plantarum* 299v) (DSM 9843) is a probiotic strain able to reside in the human colonic mucosa *in vivo*, with an antibacterial activity against several potential pathogenic agents and an immunomodulatory activity *via* an increased interleukin-10 synthesis and secretion in colonic macrophages and T-cells.

Research frontiers
Recent studies have highlighted disturbances of the relationship between the complex community of the gut microbiota and their host in irritable bowel syndrome. The potential to correct this using probiotics has been suggested but the effective strains need to be determined.

Innovations and breakthroughs
After a treatment of 4 wk, the relief or improvement of irritable bowel syndrome (IBS) symptoms was greater with the *L. plantarum* 299v group than with placebo (P < 0.05) leading to greater patient satisfaction.

Applications
L. plantarum 299v (DSM 9843) is a suitable candidate for the relief of moderate symptoms in any IBS patient.

Peer review
Overall this is a well written paper reporting a trial of reasonable methodology. It should be published if authors can revise it in a satisfactory manner.

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BRIEF ARTICLE

Incidental gallbladder cancer during laparoscopic cholecystectomy: Managing an unexpected finding

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Abstract

AIM: To evaluate the impact of incidental gallbladder cancer on surgical experience.

METHODS: Between 1998 and 2008 all cases of cholecystectomy at two divisions of general surgery, one university based and one at a public hospital, were retrospectively reviewed. Gallbladder pathology was diagnosed by history, physical examination, and laboratory and imaging studies [ultrasonography and computed tomography (CT)]. Patients with gallbladder cancer (GBC) were further analyzed for demographic data, and type of operation, surgical morbidity and mortality, histopathological classification, and survival. Incidental GBC was compared with suspected or preoperatively diagnosed GBC. The primary endpoint was disease-free survival (DFS). The secondary endpoint was the difference in DFS between patients previously treated with laparoscopic cholecystectomy and those who had oncological resection as first intervention.

RESULTS: Nineteen patients (11 women and eight men) were found to have GBC. The male to female ratio was 1:1.4 and the mean age was 68 years (range: 45-82 years). Preoperative diagnosis was made in 10 cases, and eight were diagnosed postoperatively. One was suspected intraoperatively and confirmed by frozen sections. The ratio between incidental and nonincidental cases was 9/19. The tumor node metastasis stage was: pTis (1), pT1a (2), pT1b (4), pT2 (6), pT3 (4), pT4 (2); five cases with stage I a (T1 a-b); two with stage I b (T2 N0); one with stage II a (T3 N0); six with stage II b (T1-T3 N1); two with stage III (T4 Nx Nx); and one with stage IV (Tx Nx Mx). Eighty-eight percent of the incidental cases were discovered at an early stage ($\leq$ II). Preoperative diagnosis of the 19 patients with GBC was: GBC with liver invasion diagnosed by preoperative CT (nine cases), gallbladder abscess perforated into hepatic parenchyma and involving the transversal mesocolon and hepatic hilum (one case), porcelain gallbladder (one case), gallbladder adenoma (one case), and chronic cholelithiasis (eight cases). Every case, except one, with a T1b or more advanced invasion underwent IVb + V wedge liver resection and pericholedochic/hepatoduodenal lymphadenectomy. One patient with stage T1b GBC refused further surgery. Cases with Tis and T1a involvement were treated with cholecystectomy alone. One incidental case was diagnosed by intraoperative frozen section and treated with cholecystectomy alone. Six of the nine patients with incidental diagnosis reached 5-year DFS. One patient reached 38 mo survival despite a port-site recurrence 2 years after original surgery. Cases with non in-

cidental diagnosis were more locally advanced and only two patients experienced 5-year DFS.

CONCLUSION: Laparoscopic cholecystectomy does not affect survival if implemented properly. Reoperation should have two objectives: R0 resection and clearance of the lymph nodes.

Key words: Incidental gallbladder cancer; Laparoscopic cholecystectomy; Lymph nodes; Hepatic resection; Management; Outcome

INTRODUCTION

The widespread use of laparoscopic techniques has led to an increase in referrals for cholecystectomy. As a consequence, the incidental finding of gallbladder cancer (GBC) at an earlier stage has altered the management and the outcome of the disease. However, GBC remains a lethal disease associated with a dismal prognosis. Controversies exist on the optimal treatment of this unexpected finding during routine laparoscopic cholecystectomy. The management is difficult because no guidelines have been established and some authors have reported worse overall prognosis when the patient was not adequately treated during the first operation. If GBC is suspected preoperatively, open cholecystectomy must be performed to enable a complete evaluation of the disease extent and to allow radical resection, if necessary.

Simple cholecystectomy may be adequate treatment only for the earlier stages: Tis and T1a. Reoperation is recommended in cases of T2 tumors and more advanced stages of disease. On the contrary, controversies still exist on the need for more radical resection for T1b GBC. During reoperation it is also unclear what the appropriate extent of hepatic resection is, and whether hepatic resection can prevent liver recurrence.

We report our 10 years experience (19 cases) in the treatment of GBC, and we present a systematic review to evaluate the role of extended surgery in the treatment of the incidental GBC. A Medline search was performed using the keywords "Incidental gallbladder cancer", "laparoscopic cholecystectomy", "lymph nodes dissection" and "hepatic resection".

Reviewing the literature, we focused on the following key points, which are still considered controversial in the management of GBC: (1) How laparoscopy has modified the presentation, the outcome, and the management of the patients with gallbladder cancer? (2) What is an appropriate extent of hepatic resection during reoperation, and can hepatic resection prevent liver recurrence? (3) What is the optimal extent of lymph node dissection? (4) When is resection of the common bile duct necessary? (5) Which type of surgical strategy should be used according to depth invasion? (6) Does laparoscopic cholecystectomy worsen prognosis? (7) Are port-site metastases a real problem? and (8) When is additional radical resection not indicated?

MATERIALS AND METHODS

Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All patients provided informed consent.

Data collection

From 1998 to 2008, in the Department of General Surgery of Catania University Hospital and in the General Surgery Unit of Taormina Hospital, 1490 patients underwent cholecystectomy. Within this group of patients, all the cases of GBC were retrospectively reviewed. Patients' demographic data, as well as type of operation, surgical morbidity and mortality, histopathological classification, and survival data were collected in a database for further analysis. The diagnosis of gallbladder pathology was made by history, physical examination, and laboratory and imaging studies [ultrasonography and computed tomography (CT)].

Disease-free survival analysis

The patients were divided in two groups: incidental diagnosis of gallbladder carcinoma, and known or suspected diagnosis preoperatively. The primary endpoint of the study was disease-free survival (DFS) at different stages of diagnosis. The secondary endpoint was the difference in DFS between patients previously treated with laparoscopic cholecystectomy and patients who had oncological resection as their first intervention. The results are reported in percentages and means.

RESULTS

GBC was diagnosed in 19 patients, 11 women and eight men. The male to female ratio was 1:1.4 and the mean age was 68 years (range: 45-82 years).

According to tumor node metastasis staging of the 6th edition of the American Joint Committee on Cancer (AJCC), our patients were divided into: pTis (1), pT1a (2), pT1b (4), pT2 (6), pT3 (4), pT4 (2); five cases with stage I a (T1 a-b); two with stage I b (T2 N0); one with stage II a (T3 N0); six with stage II b (T1-T3 N1); two

Table 1 Patient characteristics with gallbladder cancer _n_ (%)

	IGBC	NIGBC
No. of patients (_n_ = 19)	9	10
Polyposis lesions	7 (77.8)	5 (50)
Nonpolyposis lesions	1 (11.1)	5 (50)
Histopathological grade		
G1	3 (33.3)	1 (10)
G2	6 (66.7)	3 (30)
G3	0	6 (60)
Lymphatic invasion		
+	2 (22.2)	4 (40)
-	7 (77.8)	6 (60)
Vessel invasion		
+	1 (11.1)	1 (10)
-	8 (88.9)	9 (90)
Perineural invasion		
+	1 (11.1)	3 (30)
-	8 (88.9)	7 (70)
Stage		
0	1 (11.1)	0
I A	4 (44.4)	2 (20)
I B	1 (11.1)	1 (10)
II A	0	1 (10)
II B	2 (22.2)	4 (40)
III	0	1 (10)
IV	1 (11.1)	1 (10)

IGBC: Incidental gallbladder cancer; NIGBC: Nonincidental gallbladder cancer. +: Positive; -: Negative.

with stage III (T4 Nx Nx); and one with Stage IV (Tx Nx Mx). Eighty-eight percent of the incidental cases were discovered at an early stage ($\leqslant$ II). A preoperative diagnosis was possible only in 10 cases; eight were diagnosed postoperatively during the pathological examination; and one was suspected intraoperatively and then confirmed by frozen sections. The ratio between incidental and nonincidental cases was 9/19, with eight cases discovered after laparoscopic cholecystectomy. The preoperative diagnosis of the 19 patients with GBC was: GBC with liver invasion diagnosed by preoperative CT (nine cases); gallbladder abscess perforated into hepatic parenchyma and involving the transversal mesocolon and hepatic hilum (one case); porcelain gallbladder (one case); gallbladder adenoma (one case); and chronic cholecystolithiasis (eight cases).

Pathological characteristics of the tumors were: one *in situ* cancer; three well-differentiated polypoid adenocarcinoma (G1); one well-differentiated nonpolypoid adenocarcinoma of the gallbladder fundus (G1); seven moderately differentiated polypoid adenocarcinoma (G2-G3); one moderately differentiated nonpolypoid adenocarcinoma (G2); and one and five polypoid and nonpolypoid poorly differentiated GBC (G3), respectively (Table 1).

Every case, except one, with a T1b or more advanced invasion underwent IVb + V wedge liver resection and pericholedochic/hepatoduodenal lymphadenectomy. One patient with stage T1b refused further surgery. Cases with Tis and T1a involvement were treated with cholecystectomy alone. One incidental case was diagnosed by intraoperative frozen section and treated with cholecystectomy alone. Six of the nine patients with in-

cidental diagnosis reached 5-year DFS. Surprisingly, one patient reached 38 mo survival despite a port-site recurrence 2 years after the original surgery requiring further resection. Cases with nonincidental diagnosis were more locally advanced and only two patients experienced 5 years DFS (Tables 2 and 3).

DISCUSSION

How laparoscopy has modified presentation, outcome and management of patients with GBC

Presentation and outcome: The widespread use of laparoscopic cholecystectomy has led to discovery of this deadly disease at an earlier stage, altering the management and the outcome of these patients. GBC is an incidental finding in 0.25%-3% of patients and almost half of these cases are occasionally discovered during or after laparoscopic cholecystectomy for benign disease, such as gallstones and their complications (47% in the series of Memorial Sloan-Kettering Cancer Centre, 50% in the series of Johns Hopkins)[1,2]. The earlier discovery results in an earlier pathological stage, and consequently, increased long-term survival[2-4]. Patients with incidental GBC had a significant increase in survival when compared with those who had a preoperative diagnosis (overall 5-year survival 15% *vs* 33%)[2]. Therefore, the general surgeon should be prepared to deal with GBC suspected or diagnosed incidentally, following a well-established treatment algorithm[5-8]. It is paramount not to violate oncological principles during the first operation, if a two-stage approached is necessary. For this reason, the surgeon during video-laparoscopic cholecystectomy should always follow these simple rules: (1) perform a thorough preoperative diagnosis; (2) when in doubt, give up the laparoscopy to open access; (3) try to preserve the integrity of the gallbladder, handling it as little as possible; (4) close possible breaches of the wall with clips or endoloops; (5) always use the endobag for the removal of the gallbladder; (6) carefully inspect the gallbladder once extracted; (7) if in doubt, perform a histological examination impromptu; and (8) desufflate the pneumoperitoneum with the trocars *in situ*. During cholecystectomy, accidental opening of the gallbladder is described in 25%-30% of the cases, which clearly have a worse prognosis[3,9].

Management: The approach to incidental GBC is still controversial because of the difficulty of comparing data deriving from nonuniform case studies. Particularly discordant are the data deriving from western cancer registries with respect to the Japanese ones[3,4,10-13]. The only constant element seems to be that the prognosis strongly depends on the stage and on the possibility of achieving R0 oncological resection[3,4]. When incidental GBC is diagnosed afterwards by the pathologist, it is essential to restage the patients carefully by CT, magnetic resonance imaging and positron emission tomography, with a targeted study of the liver bed, peritoneum and of orifices of the trocars[14,15]. Moreover, a reassessment of

Table 2 Patient characteristics: Demographic data, histopathological classification, tumor node metastasis staging

Patient	Gender	Age (yr)	Incidental	TNM 6th edition	Cystic duct	Resection	Size (mm)	Grade	Lymphatic	Vessel	Perineural	5-yr survival
1	M	63	No	pT2 N1 Mx	R0	R0	10 (NP)	G3	No	R0	No	Alive, 15 mo
2	F	82	No	pT4 N2 M1	R0	R1	45 (NP)	G3	Yes	Yes	Yes	Dead, 3 mo
3	F	60	No	pT3 N1 Mx	R1	R1	60 (P)	G3	No	No	Yes	Dead, 6 mo
4	F	72	No	pT3 N1 Mx	R0	R1	32 (NP)	G3	Yes	No	No	Dead, 8 mo
5	M	76	No	pT4 N1 Mx	R0	R1	49 (NP)	G3	Yes	No	Yes	Dead, 7 mo
6	M	81	No	pT3 N0 Mx	R0	R1	44 (NP)	G3	No	No	No	Dead, 9 mo
7	F	77	No	pT2 N0 Mx	R0	R0	20 (P)	G2	No	No	No	Dead, 24 mo
8	F	45	No	PT1a N0 Mx	R0	R0	25 (P)	G1	No	No	No	Alive, no recurrence at 5 yr
9	F	81	No	PT3 N1 Mx	R0	R1	24 (P)	G2	Yes	No	No	Dead, 28 mo
10	F	66	No	pT1b N0 Mx	R0	R0	7 (P)	G2	No	No	No	Alive, no recurrence at 5 yr
11	M	69	Yes	pT1b N0 Mx	R0	R0	15 (NP)	G1	No	No	No	Alive, 38 mo (disease recurrence)
12	M	65	Yes	PT1a Nx Mx	R0	R0	18 (P)	G1	No	No	No	Alive, no recurrence at 6 yr
13	F	72	Yes	pT2 N0 Mx	R0	R0	10 (NP)	G2	No	No	No	Alive, no recurrence at 5 yr
14	M	55	Yes	pT2 N0 M1	R1	R1	30 (P)	G2-3	No	Yes	Yes	Dead, 8 mo
15	F	78	Yes	pT2 N1 Mx	R0	R0	14 (P)	G2-3	Yes	No	No	Dead, 26 mo
16	F	57	Yes	pT1b N0 Mx	R0	R0	30 (P)	G2-3	No	No	No	Alive, no recurrence at 5 yr
17	M	71	Yes	pT2 N1 Mx	R0	R0	20 (P)	G2-3	Yes	No	No	Dead, 23 mo
18	F	61	Yes	pTis Nx Mx	R0	R0	12 (P)	G1	No	No	No	Alive, no recurrence at 5 yr
19	M	69	Yes	pT1b N0 Mx	R0	R0	5 (P)	G2	No	No	No	Alive, no recurrence at 5 yr

TNM: Tumor node metastasis; M: Male; F: Female; NP: Non polypoid; P: Polypoid.

the histological examination has to be performed, with a possible second opinion. This is important in order to: (1) confirm the pT; (2) specify the exact site of the tumor (hepatic side, bottom, infundibulum); (3) have a thorough evaluation of the cystic duct; and (4) evaluate whether the cystic lymph node is included in the histological examination. Today reoperation for incidental GBC should have two fundamental objectives: R0 resection of the liver parenchyma with the other adjacent structures, and clearance of the locoregional lymph nodes[7,8].

What is an appropriate extent of hepatic resection during reoperation and can hepatic resection prevent liver recurrence?

Hepatic resection for GBC must have two main aims: resect the tumor that has directly invaded the liver from the gallbladder bed, and prevent micrometastases that may recur around the gallbladder bed[3]. However, it remains unclear what an appropriate extent of hepatic resection is, and whether hepatic resection can prevent liver recurrence. Generally, operative procedures for incidental GBC include: extended cholecystectomy or Glenn resection (i.e., cholecystectomy plus partial resection of liver segments 4 and 5, approximately 2-3 cm from the gallbladder bed); anatomic resection of liver segment 5 and lower part of segment 4 when GC invades the liver bed to a depth of 2 cm or more; right hepatectomy when GC invaded the right Glisson capsule[3,7,8].

As noted in the literature, the preference today is for parenchyma-sparing operations, such as no anatomical wedge resection[1,3]. Araida *et al*[16] showed in a multi-center retrospective study, that there was no significant differences in survival and in recurrence rates of liver metastasis between patients that underwent resection of the gallbladder bed, anatomical segmentectomy 4b + 5 and hepatectomy for pT2 and pT3 GBC. He also proved that there were no particular preferences of recurrent liver metastasis for segment 4a + 5. Similarly, other authors have reported that there was no association between major hepatectomy and long-term survival, and that there was an increased association between major hepatic surgery and perioperative morbidity[1,3].

In order to support this, Pawlik *et al*[5] have proved that patients who had undergone major hepatic resection (anatomical segmentectomy of 4a + 5 or hemihepatectomy) had a similar risk of specific death as patients who underwent hepatic wedge resection, on both univariate and multivariate analyses. Rather than the type of hepatic resection, the most important factor that determines the final outcome is to obtain R0 resection. In fact, R1/R2 margin status is associated with decreased long-term survival[17].

In conclusion, for gallbladder cancer without hepatoduodenal ligament invasion and without any locoregional liver involvement, the wedge resection of the gallbladder bed (3 cm) is preferable to hepatectomy[3,5,17]. With regard to GBC that has invaded the gallbladder bed, in order to obtain negative histological margins, the preferred approach is nonanatomical resection of hepatic parenchyma, with a distal clearance of at least 2 cm[3,5,17].

Table 3 Patient characteristics: Type of operation and survival data

Patient	Gender	Age (yr)	Incidental	TNM 6th edition	Cystic duct	Resection	Surgery	5-yr survival
1	M	63	No	pT2 N1 Mx	R0	R0	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Alive, 15 mo
2	F	82	No	pT4 N2 M1	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage) + CBD res.	Dead, 3 mo
3	F	60	No	pT3 N1 Mx	R1	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 6 mo
4	F	72	No	pT3 N1 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 8 mo
5	M	76	No	pT4 N1 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 7 mo
6	M	81	No	pT3 N0 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 9 mo
7	F	77	No	pT2 N0 Mx	R0	R0	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 24 mo
8	F	45	No	PT1a N0 Mx	R0	R0	Cholecystectomy, no further surgery	Alive, no recurrence at 5 yr
9	F	81	No	PT3 N1 Mx	R0	R1	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Dead, 28 mo
10	F	66	No	pT1b N0 Mx	R0	R0	Wedge res. (IVb + V) + lymphadenectomy (I stage)	Alive, no recurrence at 5 yr
11	M	69	Yes	pT1b N0 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Alive, 38 mo (disease recurrence)
12	M	65	Yes	PT1a Nx Mx	R0	R0	Cholecystectomy	Alive, no recurrence at 6 yr
13	F	72	Yes	pT2 N0 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Alive, no recurrence at 5 yr
14	M	55	Yes	pT2 N0 M1	R1	R1	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + CBD and PS exc	Dead, 8 mo
15	F	78	Yes	pT2 N1 Mx	R0	R0	Cholecystectomy, refused further surgery	Dead, 26 mo
16	F	57	Yes	pT1b N0 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Alive, no recurrence at 5 yr
17	M	71	Yes	pT2 N1 Mx	R0	R0	LC (stage) - wedge res. (IVb + V) + lymphadenectomy (II stage) + PS exc	Dead, 23 mo
18	F	61	Yes	pTis Nx Mx	R0	R0	LC	Alive, no recurrence at 5 yr
19	M	69	Yes	pT1b N0 Mx	R0	R0	Cholecystectomy, refused further surgery	Alive, no recurrence at 5 yr

TNM: Tumor node metastasis; M: Male; F: Female; CBD: Common bile duct; PS: Port site; LC: Laparoscopic cholecystectomy; res: Resection of segments; exc: Excision.

Optimal extent of lymph node dissection?

In GBC, besides radical R0 resection, another main aim of surgery is to obtain complete clearance of the locoregional lymph nodes. GBC spreads through different pathways: direct locoregional invasion to lymphatic, vascular and neural invasion. The most common route of dissemination is lymphatic diffusion. This is facilitated by lymphatic channels in both the muscular and subserosal layers of the gallbladder. In addition, neoplastic cells, even without evident transmural invasion, often spread superficially to the other lymph nodes along the bile ducts[18-20]. The lymph nodes involved in the locoregional spread of GC can be divided into three: (1) cystic, pericholedochal and hilar lymph nodes; (2) lymph nodes around the portal vein, the common hepatic artery and periduodenal and peripancreatic lymph nodes; and (3) celiac, superior mesenteric artery and the para-aortic lymph nodes[18-20].

Although the cystic and pericholedochal lymph nodes are the first key station, the pathways of lymph node involvement from the first site of diffusion to the hepatoduodenal ligament (cystic, pericholedochal and hilar lymph nodes) tend to be highly variable[21]. In fact, GBC can spread directly to the third level of lymph nodes, along the perivascular soft tissue (celiac, superior mesenteric artery and the para-aortic lymph nodes), according to the three pathways of lymphatic drainage proposed

by Ito *et al*[21]: cholecysto-retropancreatic pathway (main pathway), cholecysto-celiac and cholecysto-mesenteric pathways (accessory pathways). The incidence of occult lymphatic metastasis discovered during reoperation for incidental GBC can vary from 0% to 85% in relation to the depth of organ invasion (pT).

In fact, the reported incidence of occult lymphatic metastasis by stage is as follows: for T1a 0%-2.5%, for T1b 15%-25%, for T2 30%-50%, for T3 45%-75%, and for T4 > 85%[5,18,22-26] (Table 4). Similarly to other cancers, lymphadenectomy not only provides important staging information, but more importantly, may decrease the risk of locoregional recurrence. In fact, after tumor resection, the level of lymph node metastasis correlates with overall prognosis within the same pT stage category[25,26]. Miyakawa *et al*[27] reported 5-year survival of 60.3% for pN0 patients, 30.0% for pN1, 16.8% for pN2, and 5.9% for pN3. Hence, little controversy exists on the optimal management of T1a GBC. In fact, cholecystectomy alone is sufficient[3]. On the contrary, controversy still exists on the need for more radical resection in T1b GBC[24,26]. Moreover, different authors have advocated that not all T1b stages are the same, and treatment should be individualized. In fact, due to the strong correlation between lymphatic invasion and lymph node metastasis, Shibata *et al*[20] have advocated the use of

Table 4 Residual disease in the lymph nodes after re-resection for each pT (%)

Pathological T	Ogura *et al*[22]	Tsukada *et al*[18]	Foster *et al*[23]	Pawlik *et al*[5]	You *et al*[24]	Liang *et al*[25]	Erich *et al*[26]
Tis							
T1a	2.5	0			0	0	0
T1b	15.5	0			3.8	0	24.4
T1 (tot)	18	0		12.5	3.8	0	24.4
T2	44.3	46	33	31.3		29.2	44.9
T3	72		75	45.5		58.7	63.7
T4						85.36	

lymphatic invasion as guidance for additional radical resection. However, this remains controversial because the absence of lymph node invasion does not exclude other recurrence such as liver metastases, peritoneal carcinomatosis or recurrence at the port sites, or expression of other forms of diffusion of GBC[28,29].

Based on our review, we believe that resection of the gallbladder bed with regional lymph node dissection is the best choice for treatment of T1b GBC. In Western countries, lymphadenectomy is usually confined to the hepatoduodenal ligament around the hilar area (N1 lymph nodes: cystic, pericholedochal and hilar lymph nodes). Extended radical lymphadenectomy of N2 lymph nodes (including lymph nodes around the portal vein, common hepatic artery, and periduodenal and peripancreatic lymph nodes) is not routinely advocated[3,4]. Currently, according to the 7th edition of AJCC staging, N2 involvement is considered as M1 metastasis, and represent a potential contraindication to additional radical surgery[30].

When is resection of the common bile duct necessary?

Resection of the common bile duct performed at the time of the hepatic resection and lymphadenectomy is controversial[31-33]. GC has a strong tendency to invade the hepatoduodenal ligament in the form of perineural invasion or lymph node metastasis, therefore, *en bloc* resection of the regional lymph nodes together with excision of the connective tissue around the portal and hepatic artery should be performed, whenever lymph node dissection of the hepaduodenal ligament is entertained[34-36]. Dissection of the hepatoduodenal ligament implies a risk of inducing ischemic damage to the common bile duct, therefore, Shimizu *et al*[10] proposed routine resection of the extrahepatic bile duct to facilitate lymphadenectomy, avoiding common bile duct ischemia, and increasing the number of lymph nodes harvested. However, these benefits have not been confirmed in other studies[32,33]. Pawlik *et al*[5] showed that the median number of lymph nodes harvested at the time of lymphadenectomy was the same ($n = 3$), regardless of whether the common bile duct was or was not resected concomitantly with lymph node dissection ($P = 0.35$). Araida *et al*[31] found that, in patients with advanced GBC, who did not have direct invasion of the hepatoduodenal ligament and/or of the cystic duct, bile duct resection did not result in any differences in terms of recurrence and overall survival, but it only exposes patients to the potential complications of the bilioenteric anastomosis.

In conclusion, bile duct resection should be performed only when the patients have a positive involvement of the cystic duct margins, discovered either on the pathological review of the initial cholecystectomy or through biopsy of the cystic duct at the time of the second operation[3,32,33]. In fact, microscopic involvement of the cystic duct margin is associated with a residual and/or additional disease in the common bile duct in over one-third of the cases[32,33].

Type of treatment according to depth invasion

Contrary to other gastrointestinal carcinomas, the depth of invasion of GBC dictates the extent of surgical resection. In cases of carcinoma *in situ* or tumor invading the mucosa (Tis and T1a), simple cholecystectomy with negative surgical margins can be considered as curative surgery[3,4,23,37].

The 5-year survival after simple cholecystectomy is between 99% and 100%[23,37]. When the muscularis layer is involved (T1b), a 20%-50% local-regional recurrence can be expected after simple cholecystectomy[3,6,37] (Table 5). At the time of reoperation, it has been shown that there is a 10% incidence of residual disease in the liver bed associated with a 15%-25% incidence of residual metastatic lymph node involvement[22,24,26]. The 5-year survival after simple cholecystectomy is between 40% and 50%[6,23,36-39]. Therefore, the recommended procedure is cholecystectomy associated with resection of at least 3 cm of liver parenchyma (wedge resection), plus adequate lymphadenectomy (Glenn's resection)[37-39]. When the tumor extends beyond the serosa and invades the liver or an organ or an adjacent structure (T3), there is a 36% incidence of residual disease at the liver level and 45%-75% incidence of lymph node dissemination[5,22,25,26]. The goal of surgical intervention is to obtain R0 resection, hence, mandatory steps include extended lymphadenectomy and extended hepatic resection, associated with resection of other organs and structures, when necessary[35]. T3 patients are at high risk of peritoneal metastases, therefore, explorative laparoscopy should be considered in order to avoid unnecessary laparotomy. The 5-year survival after simple cholecystectomy is 0%-15%, and reaches 25%-65% after extended resection[5,23,36] (Table 5).

Does laparoscopic cholecystectomy worsen prognosis?

More cases of GBC are incidentally diagnosed during laparoscopic cholecystectomy, thus, the question arises whether laparoscopic cholecystectomy worsens the prog-

Table 5 Five-year survival according to both stage of gall-bladder cancer and type of surgery (%)

Author	T1a LC	T1b LC	T2 LC	T2 extended resection
Fong *et al*[36]			19	61
Wagholikar *et al*[37]	100	41.67		
Fong *et al*[38]			20	60
Foster *et al*[23]	100	50	38	78
Chijiiwa *et al*[39]			17	75

LC: Laparoscopic cholecystectomy.

Table 6 Metastasis at port-site and at subcostal laparotomy (%)

Author	Metastasis at port-site	Metastasis at subcostal laparotomy
Z'graggen *et al*[43]	14	
Wu *et al*[44]	16	6.5
Paolucci *et al*[47]	17.1	
Paolucci *et al*[48]	14	12

nosis of these patients. Drouard *et al*[40] first described the development of port-site metastases in 1991, and additional proof came in 1994[41]. This contributed to the loss of interest in approaching malignancy laparoscopically. Furthermore, excessive manipulation of the organ and perforation can cause intraperitoneal spread of malignant cells, resulting in a worse long-term survival[42]. In fact, the incidence of port-site recurrence increased from 9% in patients without intraoperative perforation to 40% in those in whom perforation could be demonstrated[43]. Other studies proved that pneumoperitoneum significantly increased tumor cell implantation at trocar sites, and tumor growth in the peritoneum[44-46]. However, laparoscopic cholecystectomy, if correctly performed, did not influence the long-term prognosis of early stage tumors (T1a, T1b, T2)[7,8]. Also, radical re-resection, performed several months after laparoscopic cholecystectomy, has similar results to radical resection in one stage, and long-term survival can be achieved in tumors with infiltration of the liver in patients who have previously undergone noncurative surgery[1,7,8,23]. Survival is strictly related to the depth of parietal invasion of the tumor, but there is no significant difference between patients with incidental GBC discovered during or after cholecystectomy ($P = 0235$)[7]. The real problem is to have a clear understanding of how to deal with this eventuality.

Are port-site metastasis a real problem?

Port-site metastasis is the most common form of parietal recurrence (Table 6). It has been reported at all stages of gallbladder carcinoma and at any of the trocar sites. It generally presents after latency, ranging from a few months to 3-4 years. Many factors can contribute to port-site metastasis. One of the most important is intraoperative spillage of bile from gallbladder wall perforation, which has been described in 30% of laparoscopic cholecystectomy cases, and it has been linked to port-site metastasis[43,44,47,48]. Intraoperative manipulation of the tumor, in the form of tension, dissection and isolation, often leads to the disintegration of a certain proportion of cancer cells, as confirmed by the presence of granular cells in 40% of laparoscopic instruments[49,50]. The increased intraperitoneal pressure induced by the CO_2 pneumoperitoneum can spread and redistribute cancer cells within the peritoneal cavity and in damaged surfaces. Finally, evidence exists on the immunosuppressive action of CO_2 which would favor the implantation of tumor

cells[50]. The median survival after port-site metastasis is approximately 1 year, and it is mandatory to perform resection at the time of reintervention in patients previously treated with laparoscopic cholecystectomy[3,7,8].

Contraindications to additional radical resection

With the primary goal of surgery in mind (R0 resection), the only contraindication to additional surgery is the inability to obtain radical R0 resection. In particular, the presence of peritoneal metastasis, distant metastasis, locally advanced GBC with N2 or M1 (according to the 7th edition of AJCC staging), lymph node invasion along the hepatic artery, portal vein and celiac and mesenteric vessels are all considered contraindications to radical resection[35,51-53]. On the other hand, the presence of peripancreatic (head only) lymph node disease is not a contraindication to surgical excision, and radical lymphadenectomy and pancreaticoduodenectomy can be carried out together with liver resection[35,53]. Also, the depth of liver involvement and multiorgan locoregional involvement do not represent a contraindication for additional radical resection[51,52]. Combined pancreaticoduodenectomy, right hemicolectomy and major hepatectomy are effective treatment for GBC with direct invasion of the adjacent organs (stomach, duodenum, pancreas, colon and liver), but only if potentially curative resection (R0) is feasible. In these cases of multiorgan resection for GBC, given radical R0 resection, the long-term survival will depend on bile duct involvement[35,51-53]. In fact, stromal invasion of the extrahepatic bile ducts is sometimes a prelude to hepatoduodenal ligament involvement, and is also associated with a higher rate of metastases to para-aortic nodes with a high incidence of residual tumor and poor outcome after surgery[32].

In conclusion, incidental carcinoma of the gallbladder, as our experience confirms, generally is diagnosed at an earlier stage and carries a better prognosis than non-incidentally found cancer. Laparoscopic cholecystectomy does not affect survival if implemented with proper technique. Simple cholecystectomy may be an adequate treatment only for earlier stage GBC: Tis and T1a. All other stages, starting from T1b should be treated with lymphadenectomy and resection of at least 2-3 cm of liver parenchyma around the liver bed, provided that no residual microscopic cancer (R0) remains. Resection of the main bile ducts could be necessary in hilum-type cancers with positive margins of the cystic duct. More extensive liver resection or performance of multiorgan resection can be pursued in order to achieve R0 resection.

COMMENTS

Background

Gallbladder carcinoma remains a rare, but highly aggressive disease. Its dismal prognosis is associated with the advanced stage of the disease at the time of diagnosis.

Research frontiers

Controversy exists about the optimal management of the disease. In particular, the debate involves the extent of surgical resection of the liver and surrounding organs, the need for resection of the main bile duct, the extent of lymph node removal, and the potential for negative effects of previous laparoscopic cholecystectomy. It is also unclear when surgery is not indicated at all.

Innovations and breakthroughs

Previous studies have proved how simple cholecystectomy is sufficient treatment for early stages of gallbladder carcinoma. Also, it seems that, at more advanced stages, it is paramount to obtain complete gross oncological resection (R0) without the need for anatomical hepatic resection. In order to minimize port-site metastasis, the laparoscopic approach to apparently benign gallbladder disease has to follow specific principles: minimal manipulation of the gallbladder; avoidance of rupture of the gallbladder and bile spillage; extraction of the specimen with a protective bag to avoid contact with the skin; and evacuation of the intraperitonealy insufflated gas *via* the cannulae.

Applications

The authors conclude that gallbladder cancer can be adequately cured when the diagnosis is early and the treatment is standardized by stage. Incidental carcinoma of the gallbladder is generally diagnosed at an earlier stage and carries a better prognosis than nonincidentally found cancer. Laparoscopic cholecystectomy does not affect survival if implemented with proper technique. Simple cholecystectomy may be adequate treatment only for the earlier stages. All other stages should be treated with lymphadenectomy and resection of at least 2-3 cm of liver parenchyma around the liver bed, provided that no residual microscopic cancer remains. Resection of the main bile ducts could be necessary in cancers with positive margins of the cystic duct. More extensive liver resection or performance of multiorgan resection can be pursued in order to achieve complete resection of the tumor. This research can certainly guide surgeons that encounter this rare entity unexpectedly. In fact, as long as the appropriate referrals are made, the prognosis does not worsen. This can also increase awareness of this rare, but potentially lethal disease.

Terminology

R0 is the surgical removal of all the grossly apparent tumor cells. Port-site metastasis refers to implantation of tumor cells at the skin incisions utilized to place the laparoscopic trocars.

Peer review

The authors present solid experience with a rare disease. Their clinical analysis, along with a thorough review of the literature, provides a clear algorithm to approach the disease at different stages.

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BRIEF ARTICLE

Matrix metalloproteinases in the restorative proctocolectomy pouch of pediatric ulcerative colitis

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Abstract

AIM: To investigate matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in pouch mucosa of pediatric onset ulcerative colitis (UC).

METHODS: In this cross-sectional study, 28 patients with pediatric onset UC underwent ileal pouch biopsy 13 years (median) after proctocolectomy. Expression of MMPs-3, -7, -8, -9, -12 and -26 and TIMPs-1, -2 and -3 in samples was examined using immunohistochemichal methods, and another biopsy was used to evaluate the grade of histological inflammation. Two investigators independently graded the immunohistochemical specimens in a semiquantitative fashion, using a scale marking staining intensity as follows: 0 = less than 20 positive cells; 1 = 20-50 positive cells; 2 = 50-200 positive cells; 3 = over 20 positive cells. Fecal calprotectin and blood inflammatory markers [serum C-reactive protein (CRP) and erythrocyte sedimentation rate] were determined during a follow-up visit to examine correlations between these markers and the expression of MMPs and TIMPs.

RESULTS: Of the 28 patients with pediatric onset UC, nine had not experienced pouchitis, whereas thirteen reported a single episode, and six had recurrent pouchitis ($\geq$ 4 episodes). At the time of the study, six patients required metronidazole. In all of the others, the most recent episode of pouchitis had occurred over one month earlier, and none were on antibiotics. Only four samples depicted no sign of inflammation, and these were all from patients who had not had pouchitis. Two samples were too small to determine the grade of inflammation, but both had suffered pouchitis, the other recurrent. No sample depicted signs of colonic metaplasia. Most pouch samples showed expression of epithelial (e) and stromal (s) MMP-3 (e, $n = 22$; s, $n = 20$), MMP-7 (e, $n = 28$; s, $n = 27$), MMP-12 (e, $n = 20$; s, $n = 24$), TIMP-2 (e, $n = 23$; s, $n = 23$) and MMP-3 (e, $n = 23$; s, $n = 28$) but MMP-8 (e, $n = 0$; s, $n = 1$), MMP-9 (e, $n = 0$; s, $n = 9$) and MMP-26 (e, $n = 0$; s, $n = 3$) and TIMP-1 ($n = 0$, both) were lacking. In samples with low grade of inflammatory activity, the epithelial MMP-3 and MMP-7 expression was increased ($r = -0.614$ and $r = -0.472$, respectively, $P < 0.05$ in both). MMPs and

TIMPs did not correlate with the markers of inflammation, fecal calprotectin, erythrocyte sedimentation rate, or CRP, with the exception of patients with low fecal calprotectin (< 100 μg/g) in whom a higher expression of epithelial MMP-7 was found no differences in MMP- or TIMP-profiles were seen in patients with a history of pouchitis compared to ones with no such episodes. Anastomosis with either straight ileoanal anastomosis or ileoanal anastomosis with J-pouch did depict differences in MMP- or TIMP-expression.

CONCLUSION: The expression of MMPs pediatric UC pouch in the long-term shares characteristics with inflammatory bowel disease, but inflammation cannot be classified as a reactivation of the disease.

INTRODUCTION

In children, ulcerative colitis (UC) appears to present more aggressively than in adults[1,2]. The number of pediatric UC cases requiring colectomy within the first decade after diagnosis may reach 24%[1]. Restorative proctocolectomy with ileoanal anastomosis (IAA) and an ileal pouchanal anastomosis is the surgical treatment of choice in UC[2].

In proctocolectomized patients, pouchitis is a common complication, and over two thirds of children undergoing proctocolectomy will suffer at least one episode of pouchitis during the following decade[2]. Yet, studies on pouchitis in children are limited. Pouchitis is an idiopathic inflammatory condition of the ileal reservoir occurring frequently after colectomy with IAA[3]. The etiology of pouchitis is poorly understood, but it is associated with risk factors such as extensive colonic disease preoperatively and young age at proctocolectomy[4]. The incidence of pouchitis is higher in UC than in familial adenomatous polyposis[5], and pouchitis has been considered a recurrent form of colitis[5,6]. Ileal pouch mucosa may acquire colonic characteristics[7,8], and colonic metaplasia is more pronounced in patients with pouchitis[9]. This mucosal transformation could have a role in the pathogenesis of pouchitis, possibly allowing the recurrence of inflammatory bowel disease (IBD) and may represent a novel manifestation IBD[7-9].

Matrix metalloproteinases (MMPs) are a family of 24 zinc-dependent enzymes implicated in mucosal damage in IBD[10]. MMPs take part in normal mucosal defense, and are capable of degrading extracellular matrix and basement membrane proteins in tissue remodeling processes both in normal and in pathological conditions[10]. In IBD, MMP-9 is the most abundantly expressed MMP, and it shows increased activity in inflamed UC mucosa[11]. Other MMPs have also been linked to inflammation severity in IBD, such as MMP-7[12]. Our group has demonstrated enhanced MMPs-1, -3 and -7 expression in IBD, and shown MMP-10 expression in IBD granulation tissue[13,14]. In pediatric IBD, we have described increased expression of epithelial MMP-10 and stromal tissue inhibitor (TIMP)-3[15].

The pathogenesis of pouchitis is unknown, and it is debated whether or not the inflammation is similar to that found in UC, or whether it is inflammation of a novel kind. The management of pouchitis by antibiotics and probiotics is widely accepted. Yet, according to a recent review, antibiotics are not significantly better than a placebo, compared to the high dose probiotics. By altering the endogenous flora and the expression of inflammation parameters, as well as competing with pathogens for receptor binding, nutrients, and growth factors, the high dose probiotics are effective in treatment of pouchitis[16]. The occurring inflammation and acquisition of colonic characteristics[7,8] inspires an interest in the MMP and TIMP-profile of pouch. We are aware of only two studies of MMP expression in pouchitis in adults[17,18]. Expression of MMP-1 and MMP-2 in pouchitis is different from normal ileal mucosa, but is similar to expression in active UC colitis[17]. We examined the MMP and TIMP profiles of pouch patients who had undergone proctocolectomy in their childhood or adolescence, and to find clues to the type of inflammation appearing in the pouch. It is important to determine whether this inflammation is IBD-like or of a novel kind, to appropriately prevent and manage pouchitis in the future.

MATERIALS AND METHODS

Patients and setting

Between 1985 and 2005, 81 pediatric patients with UC underwent proctocolectomy with ileal anastomosis at Tampere University Hospital or Children's Hospital, Helsinki. Of these colectomized patients, one died of an unrelated cause and one emigrated. Thus, 79 patients were traced from the database of the Population Register Centre and contacted by mail during 2006. Thirty-five patients agreed to participate for a follow up visit described elsewhere

Table 1 Patient characteristics

Patient characteristics	Median (range)
Number of patients (male)	28 (9)
Age (yr); median (range)	
At diagnosis	12 (1-15)
At operation	13 (2-19)
At the time of study	25 (8-41)
Duration from surgery to study	13 (4-22)
Clinical inflammation markers; mean (range)	
Fecal calprotectin (µg/g)	371 (12-2859)
ESR (mm/h)	14 (2-69)
CRP (mg/L)	3 (0-19)

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein.

Table 2 Medications at the time of study

Intestinal inflammation	n
5-ASA	1
5-ASA + antibiotics	1
Antibiotics	5
Bowel function	
To decrease bowel motility	10
To increase bulk mass	1
Analgesics	1
Lactic acid bacteria	1
Other chronic illnesses	
Cholangitis	1
Mood stabilizing agents	2
Antibiotics for acne	1
Gastro-esophageal reflux	1
Cardiac arrythmias	1
Infertility	1
Asthma	1
Hyperthyroidism	1

5-ASA: 5-Aminosalicylic acid formulations.

in detail[2], but seven had been rediagnosed as Crohn's disease (CD). The 28 UC cases were included in this study (Table 1). All proctocolectomies included transanal mucosectomy and a hand-sewn anastomosis. Twenty-one patients had an IAA with J-pouch, and 7 had straight ileoanal anastomosis (SIAA). Patients filled out a questionnaire surveying the current diagnosis, complications, medical therapy and pouchitis. To map the latter two, we asked about medications to control stool frequency, and the history and treatment of pouchitis. During the outpatient visit, biopsy of the pulled through ileum, and stool and blood samples were obtained. The Ethical Committee of the Helsinki University Hospital approved the and the participants (or their guardians) gave informed consent.

Fifteen UC patients had other chronic illnesses: sclerosing cholangitis, active hepatitis, gastroeosophageal reflux, gallstones, rheumatoid arthritis, sacroiliac-joint arthritis, knee arthritis, hyperthyroidism, cardiac arrythmias, psychiatric illness, endometriosis, fibromyalgia, osteoporosis, asthma, atopic skin and contact allergy. Specific medications at the time of the study are shown in Table 2. Postoperatively, 18 (64%) UC patients had received antibiotics

as treatment for pouchitis, and of these, 7 (25%) also had received corticosteroids.

Blood inflammatory markers and fecal calprotectin
Inflammation biomarkers in blood [erythrocyte sedimentation rate, erythrocyte sedimentation rate (ESR), and serum concentration of C-reactive protein (CRP)] and fecal calprotectin (quantitative enzyme immunoassay, PhiCal test, Calpro AS, Oslo, Norway) were routinely determined[18]. For immunohistochemistry, biopsies from pouch were used. The histological grade of inflammation in the samples was assessed according to the scoring system for pathological changes in the ileal reservoir mucosa[7,19].

Immunohistochemistry
One biopsy was analyzed for the grade of histological inflammation, the other biopsy was used for MMP immunohistochemistry. Immunohistochemistry was performed using sreptavidin-biotin-peroxidase complex technique (DakoCytomation, StreptABComplex/HRP Duet, Mouse/Rabbit, Glostrup, Denmark; and Elite goat Vectastain ABC kit, Vector laboratories, Burligame, CA, United States) or the antibody-polymer detection technique (ImmPRESS universal reagent, Anti-Mouse/Rabbit IG, Vector laboratories Burligame, CA, United States). Diaminobenzidine or NovaRED (Vector Laboratories) were used as chromogenic substrates and Mayer's hematoxylin as counterstain. Monoclonal antibodies were used to stain for MMP-7 (1:600, MAB3315, Millipore, Temecula, CA, United States)[15], MMP-8 (1:100, IM38, Calbiochem, La Jolla, CA, United States)[15], MMP-9 (1:100, MS-569-P1, Lab Vision Corporation Neomarkers, Fremont, CA, United States)[15], TIMP-1 (1:50, IM63, Calbiochem)[15], TIMP-2 (1:600, IM56, Calbiochem) and TIMP-3 (1:300, IM43L, Calbiochem)[15]. Polyclonal antibodies were used for MMP-3 (1:50, ab32607, Abcam Ltd, Cambridge, United Kingdom), MMP-12 (1:80, sc-12361, Santa Cruz Biotechnology, CA, United States)[15] and MMP-26 (1:120, a gift from Professor Isaka K, Tokyo Medical University)[15]. MMP-8 and MMP-9 were pretreated with 1% trypsin solution for 30 min at 37 ℃. MMP-3, MMP-7, MMP-26, TIMP-1, TIMP-2 and TIMP-3 were pretreated in a 95 ℃ water bath for 30 min (Dako retrieval solution pH 6; Dakocytomation). MMP-12 required no pre-treatment. The incubation conditions for the antibodies were: 4 ℃ overnight for MMPs-7, -9, -12 and TIMPs-1, -2, -3; 1 h 45 min at 37 ℃ for MMP-8 and 1 h at room temperature for MMP-3 and MMP-26. For negative controls, parallel sections of the same samples were processed using preimmune sera or normal rabbit or mouse immunoglobulin. As positive controls, we used formalin fixed, paraffin embedded sections of hailey-hailey, pyoderma gangrenosum and dermatitis herpetiformis (MMP-3), adenocarcinoma (MMP-7), squamous cell cancer (MMP-8), chronic wounds (MMP-9 and TIMP-2 and -3), foreign body reaction (MMP-12), endometrium (MMP-26), and pyoderma gangrenosum (TIMP-1). Immunohistochemical specimens were graded independently by two different investigators (Mäkitalo L, Piekkala M) in a semiquantitative fashion under a light-

Table 3 Expression profiles of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinase in ulcerative colitis samples

	Number of positive samples	mean ± SEM[1]
MMP-3		
Epithelium	22	0.93 ± 0.114
Stroma	20	1.25 ± 0.183
MMP-7		
Epithelium	28	1.36 ± 0.092
Stroma	27	1.89 ± 0.149
MMP-8		
Epithelium	0	0.00 ± 0.000
Stroma	1	0.04 ± 0.036
MMP-9		
Epithelium	0	0.00 ± 0.000
Stroma	9	0.32 ± 0.090
MMP-12		
Epithelium	20	0.75 ± 0.098
Stroma	24	1.46 ± 0.141
MMP-26		
Epithelium	0	0.00 ± 0.000
Stroma	3	0.11 ± 0.060
TIMP-1		
Epithelium	0	0.00 ± 0.000
Stroma	0	0.00 ± 0.000
TIMP-2		
Epithelium	23	0.89 ± 0.094
Stroma	23	1.75 ± 0.203
TIMP-3		
Epithelium	23	1.04 ± 0.120
Stroma	28	2.39 ± 0.130
Histology		
Neutrophils		0.85 ± 0.154
Lymphocytes		1.07 ± 0.185
Eosinophils		1.04 ± 0.167
Villus atrophy		1.19 ± 0.227
Grade		1.95 ± 0.203

[1]mean calculated from values: 0 = less than 20 positive cells; 1 = 20-50 positive cells; 2 = 50-200 positive cells; 3 = over 200 positive cells; Grade: Grade of inflammation; MMPs: Matrix metalloproteinases; TIMP: Tissue inhibitor of matrix metalloproteinase.

field microscope at × 100 magnification using a scale marking staining intensity as follows: 0 = less than 20 positive cells; 1 = 20-50 positive cells; 2 = 50-200 positive cells; 3 = over 200 positive cells[15,20]. The identity of the cell types producing each MMP or TIMP was confirmed together with an experienced pathologist (Karikoski R).

Statistical analysis

Non-parametric Mann-Whitney's test, Spearman's correlation test and independent samples t test were performed with the SPSS 17.0 for Windows (Chicago, IL) to investigate the significance of results. A $P < 0.05$ was considered significant.

RESULTS

Of the 28 patients with pediatric onset UC, nine (32.1%) had not experienced pouchitis, whereas 13 (46.4%) reported a single episode, and six (21.4%) had recurrent pouchitis (≥ 4 episodes)[19]. Six patients were on metronidazole medication at the time of the study (Table 2)

one of them also on a 5-aminosalicylic acid formulation and showed inflammation of grades 1 to 3[7,20]. In all the others the last episode of pouchitis had occurred more than one month earlier, in most cases several months earlier and they were not on antibiotics. Only four samples (14%) depicted no signs of inflammation, and these were all from patients who had not experienced pouchitis. Of the 19 patients who had experienced pouchitis, 7 showed mild inflammation, 3 depicted moderate and 7 severe inflammation in the biopsy. Two samples were too small to determine the grade of inflammation, but both had suffered pouchitis, the other recurrent. No sample depicted signs of colonic metaplasia.

Expression of MMP-3 was seen in the majority of the samples in the epithelium and in stroma in plasma cells, macrophages and eosinophils (Table 3 and Figure 1A, B). Also, MMP-3 positive endothelium was observed (Figure 1B1, B4). Epithelial MMP-7 was present in all samples, and stromal MMP-7 in 27 samples in plasma cells, macrophages and eosinophils, as well as endothelium (Table 3 and Figure 1C, D). Conversely, stromal MMP-9 was present in 9 samples in macrophages, plasma cells and eosinophils, and in intraepithelial neutrophils, but no positive enterocytes could be found (Table 3 and Figure 2D, E). MMP-12 was found in the majority of the samples in the epithelium and in stroma in macrophages, plasma cells, and eosinophils and intraepithelial neutrophils (Table 3 and Figure 2F, G1). Stromal cells showed positivity for MMP-26 in 3 samples in neutrophils and plasma cells but epithelial cells were negative for this MMP (Table 3 and Figure 2B). MMP-26 positive neutrophils were present in blood vessels (Figure 2B3). TIMP-2 was positive in the majority of samples in the epithelium and in stromal cells including plasma cells, eosinophils and macrophages (Table 3 and Figure 1E, F). TIMP-3 was found in enterocytes in 23 (82%) samples and in all samples in plasma cells and macrophages in stroma (mean 2.39) (Table 3 and Figure 1G). Endothelial cells were also positive for TIMP-3 (Figure 1G1, G2). Expression of MMP-8 and TIMP-1 was generally absent, although a few neutrophils in stroma were positive for MMP-8 and cryptal cells showed positivity for TIMP-1 (Figure 2A, C).

No differences in MMP- or TIMP-profiles were seen when comparing samples from patients that had experienced pouchitis (single or several episodes) ($n = 19$) to ones that had not ($n = 9$). The same was true when performing comparisons related to the frequency of pouchitis (data not shown). When comparing patients with SIAA to those with SIAA with J-pouch, no differences in MMP- and TIMP-expression profiles or the frequency of pouchitis episodes could be found.

MMPs and TIMPs did not generally correlate with inflammation markers fecal calprotectin, ESR and CRP. However, patients considered have no active inflammation (fecal calprotectin < 100 μg/g) showed higher expression of epithelial MMP-7 (Figure 1C) in their pouch biopsies compared to samples from those with active inflammation (fecal calprotectin ≥ 100 μg/g)[21] (Figure 1D1; means 1.75 *vs* 1.14, $P = 0.020$, respectively).
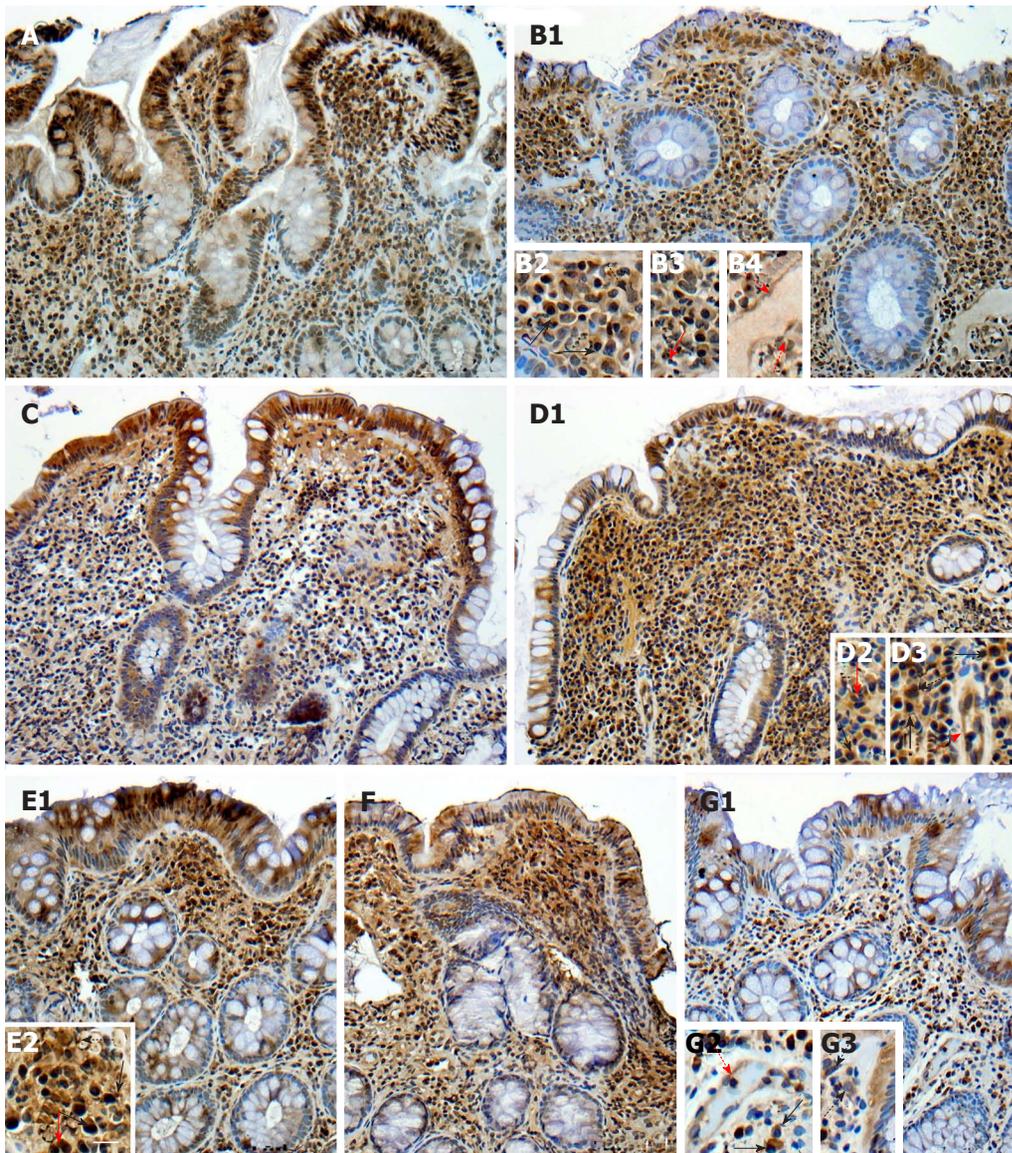
Figure 1 Matrix metalloproteinases-3, matrix metalloproteinases-7 and tissue inhibitor of matrix metalloproteinase-2 in lower (A, C, E1, respectively) and higher grade of inflammation and calprotectin levels (B1, D1, F, respectively), tissue inhibitor of matrix metalloproteinase-3 in pouch (G1). Black solid arrows plasma cells, black dotted arrows macrophages, red solid arrows eosinophils and red dotted arrows endothelium. Scale bars: 15 μm (A, B1, C, D1, E1, F, G1); 7.5 μm (B2-B4, D2, D3, E2, G2, G3). Stainings were performed using diaminobenzidine or NovaRED as chromogenic substrates and Mayer's hematoxylin as counterstain. Images were obtained using a light-field microscope, and edited using Adobe Photoshop 7.0 (Adobe Systems Incorporated).

The grade of inflammation in histological assessment[7] correlated negatively with epithelial MMP-3 (Table 4) (r = -0.614, P = 0.002) (Figure 1A, B1) and MMP-7 (r = -0.472, P = 0.027) (Figure 1C, D1).

DISCUSSION

In pediatric patients, these MMPs and TIMPs have not been studied in pouches, and in adults, reports of only MMPs 1-3 and MMP-9 exist[17,18]. This study reports for the first time expression of MMPs-7, -8, -12, TIMP-2, and TIMP-3 in ileal reservoir mucosa. In long-term, the MMP- and TIMP-profiles in pouch demonstrate similarities with IBD, but profiles in pouch cannot be said to replicate those in UC colon.

In adult inflamed and non-inflamed pouches of UC patients, MMP-3 activity is considered weak[18]. Here, MMP-3 was present in all but few samples in epithelium and stroma. In pediatric IBD, MMP-3 is elevated in endoscopically abnormal colon compared to healthy colon[22]. MMP-3 plays an important role in T-cell- and tumor necrosis factor (TNF)-α-mediated gut injury[23,24], and IBD gut plasma cells produce more MMP-3 than those of healthy controls[25]. Here, expression of epithelial MMP-3 associated with the degree of inflammation, complying with animal studies where lack of MMP-3 associated with impaired wound healing[26]. The present findings suggest that MMP-3 is an important MMP in pouch mucosa, resembling IBD. However, its relationship to the development of pouchitis is unclear. Similar to MMP-3, we found TIMP-3 in a large number of cells in stroma, expression paralleling reports in IBD colon[15,20].

Table 4 Matrix metalloproteinases and tissue inhibitor of matrix metalloproteinase that correlated with inflammation indicators

Inflammation indicators	Correlation coefficients values				
	MMP-3	MMP-7	MMP-12		TIMP-2
	Epithelium	Epithelium	Epithelium	Stroma	Epithelium
Fecal calprotectin	NS	NS	-0.350	NS	NS
C-reactive protein	NS	NS	NS	-0.422	NS
Grade of inflammation	-0.614	-0.472	NS	NS	-0.420

NS: Not significant; MMPs: Matrix metalloproteinases; TIMP: Tissue inhibitor of matrix metalloproteinase. Correlations were calculated using Spearman's correlation test.
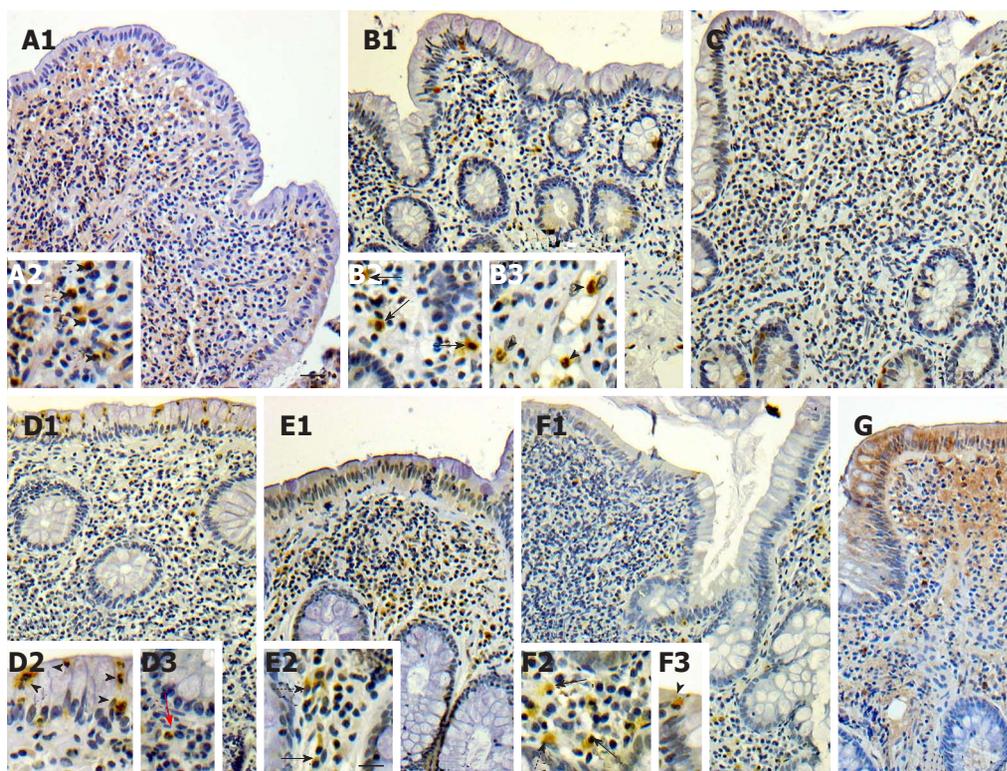
Figure 2 matrix metalloproteinases-8 (A1), matrix metalloproteinases-9 (D1, E1), matrix metalloproteinases-12 (F1, G), matrix metalloproteinases-26 (B1) and tissue inhibitor of matrix metalloproteinase-1 (C) in pouch. Inset g' added to figure from another sample, not shown in here in lesser magnification. Arrowheads depict neutrophils, black solid arrows plasma cells, black dotted arrows macrophages, red solid arrows eosinophils. Scale bars: 15 μm (A1, B1, C, D1, E1, F1,G); 7.5 μm (A2, B2, B3, D2, D3, E2, F2, F3). Stainings were performed using diaminobenzidine or NovaRED as chromogenic substrates and Mayer's hematoxylin as counterstain. Images were obtained using a light-field microscope, and edited using Adobe Photoshop 7.0 (Adobe Systems Incorporated).

Our group has noted that in intestinal ulcerations in IBD, TIMP-3 is expressed in a greater number of stromal cells compared to normal intestine[14]. TIMP-3 inhibits TNF-α[27], an important cytokine in IBD inflammation[28], and elevated TIMP-3 protein is likely to associate with elevated TNF-α-levels as seen in a human colonic cell line[29]. Also, abundant TIMP-3 expression in stroma in pouch resembles that found in pediatric UC[15], possibly preventing destructive activity as suggested for other autoimmune diseases[30].

Of the other MMPs, expression of MMP-7, MMP-8, and MMP-9 was not IBD-like. Unlike MMP-8 and MMP-9, stromal MMP-7 was present in notable numbers of cells in almost all samples. We recently examined MMP-7 expression in pediatric IBD colon, and found weaker expression in epithelium in UC samples[15]. Here, MMP-7 was found

in a smaller number of epithelial cells in samples with higher grade of histological inflammation and fecal calprotectin levels. This contradicts earlier results in UC[12,31]. However, MMP-7 contributes to intestinal wound closure but over-expression may delay epithelial wound healing[32]. Epithelial expression in pouch thus may be due to a regenerative process. MMP-7 has also been suggested to aid the growth of myofibroblasts and their function[33] and stromal MMP-7 may take part in maintaining mucosal homeostasis. In normal colon, MMP-8 is undetectable[10] but it may be involved in ulcer formation[34] as elevated levels are found in ulcer bases of both UC and CD[10]. We are not aware of previous studies of MMP-8 in pouch mucosa, but in our study, MMP-8 was generally absent from pouches. MMP-9 is important in IBD-related inflammation, and has also been shown to correlate

with disease activity[10]. We found expression of MMP-9 in only a few samples, not suggestive of IBD-like inflammation.

In our study, MMP-12 was found in almost all pouch samples, although it is not expressed in normal adult ileum[14,35]. While it's role in IBD is unknown, this MMP may take part in macrophage migration and can activate TNF-α[10,36]. MMP-12 has been proposed to be a final step in tissue injury in celiac disease[37] and might have a pathological role in pouch mucosa. Its expression suggests that pouch mucosa is dissimilar to normal ileal mucosa, even in the absence of inflammation.

In the present study, expression of MMP-26 was rare. We have proposed that immunosuppressive drugs modulate disease activity in CD by downregulating MMP-26[20]. It is found in the colon of pediatric IBD patients[15], but also in healthy ileum[38], and expression is not IBD-specific. TIMP-1 is present in greater amount in inflamed adult IBD than in healthy mucosa[31], whereas in pediatric IBD, we have reported weak TIMP-1 expression[15]. We found no expression of TIMP-1 in the pouch, but epithelial and stromal TIMP-2 was found in most samples. Structural features of TIMP-2 suggest that it is expressed in constant amount[39], and it has been reported to be present in normal-appearing IBD mucosa, with no significant increase in inflamed mucosa[40]. Expression of MMP-26, TIMP-1 and TIMP-2 were inconclusive, neither supporting of contradicting IBD-etiology.

Taken together, earlier studies on MMPs and TIMPs in pouch are scarce, but in adults elevated MMP-1 and MMP-2 have been found in pouchitis mucosa[17]. Due to small sizes of the biopsy and limited stainable slides, these MMPs were not studied here. The presence of MMP-3 and TIMP-3 in our samples is comparable to findings in IBD. In line with this, MMP-12 is detected in active IBD but not in normal ileum[14,35,41], and here, expression of MMP-12 in pouch suggests altered mucosa. The lack of proper MMP-8 and MMP-9 expression in the pouch, however, contradicts IBD-related inflammation, as does the expression profile of MMP-7.

The results here are based on immunohistochemistry only, a limitation of this study, and further research using other techniques, e.g., Western blotting and polymerase chain reaction, would be necessary to verify the findings. We could not include a side-by-side analysis of samples from active UC and normal ileum and colon. Parallel analysis of ileal and colonic samples is challenging, as the follow up lasted until early adulthood. It is possible, that the use of medication in some cases may affect the expression of MMPs and TIMPs. It has been put forth, that probiotic use - that seems to elevate interleukin-10 in the gut-might downregulate the expression of MMP activity in the pouch mucosa and alter the expression of other proinflammatory cytokines[18], but this finding needs more systematic studies. However, due to small sample size, we focused on comparing the expression patterns with established markers depicting the activity of inflammation. Interestingly, we found no differences in MMP or TIMP expression depending on the frequency of pouchitis, but most samples depicted inflammation with concurrent elevated calprotectin. It seems reasonable that since there was no variation in MMP- or TIMP-profile according to the frequency of pouchitis, that inflammation markers depict very little correlation with MMPs. While even a longer follow-up may be necessary to further investigate the nature of pouch mucosa, the strength of the study is the long follow-up of the patients with pediatric onset disease, up to two decades after surgery.

In conclusion, in long-term, MMP expression in pouch shares characteristics with UC, but inflammation cannot be classified as a reactivation *per se*. Expression of MMP-3 and TIMP-3 in pouch resembles that of IBD, but the lack of MMP-9 contradicts an IBD-like pathomechanism. To the best of our knowledge, this study is the first to show that MMPs-7, -12, and TIMP-2 are also important in pouch mucosa. Further studies on the role of MMP profiles in etiopathology and the development of pouchitis in pediatric onset UC are warranted.

ACKNOWLEDGMENTS

COMMENTS

Background
Approximately 24% of pediatric ulcerative colitis (UC) patients undergo colectomy with ileal pouch anal anastomosis within the first decade after diagnosis. Pouchitis is the most common complication occurring in approximately two thirds of the patients at least once during the following decade. The etiology of pouchitis is unknown and it is debated whether the inflammation is a novel kind or similar to that of found in UC. Matrix metalloproteinases (MMPs) have been linked to both physiological and pathological event in inflammatory bowel disease (IBD) mucosa.

Research frontiers
In pediatric IBD MMP 10 and tissue inhibitor of matrix metalloproteinase (TIMP) 3 have been linked to the colonic inflammation. Most abundantly expressed MMP in IBD-like inflammation is MMP-9 and the MMP-7 is linked to the severity of inflammation. There are no reports of MMP expression in pediatric pouch. Thus, the authors studied the expression of MMPs-3, -7, -8, -9 and -12 and TIMPs-1, -2, and -3 from the biopsies of pediatric UC patients' pouches to find MMPs and TIMPs specific to the pediatric pouchitis.

Innovations and breakthroughs
The expression of MMPs and TIMPs in pediatric pouches has not been studied previously and adult reports of only MMPs-1, -3 and -9 exist. This study, for first time, reports the epithelial and stromal expression of MMP-3, -7 -12, TIMP-2, and -3 and the lack of MMPs-8, -9, -26, and TIMP-1 expression in pediatric ileal reservoirs. The presence of MMPs-3, -12 and TIMP-3 share some similarities with IBD, but the lack of MMP-9 contradicts the IBD-like inflammation.

Applications
By understanding the MMP- and TIMP-profile of pouch mucosa, this study takes part in characterizing molecular environment in pouch, which may in turn aid in finding specific biomolecular targets for treatment of pouchitis.

Terminology
UC is chronic disease of the mucosa of the colon and the rectum characterized by bloody diarrhea, abdominal pain, and weight loss. Pouchitis is the inflammation of the ileal reservoir that is made after colectomy to avoid permanent ostomy. MMPs are a family of 24 zinc-dependent enzymes that are capable to degrade nearly all extracellular matrix and basement membrane proteins and take part to both normal and pathological tissue remodeling. TIMPs inhibit the action

of MMPs but the actions of TIMPs are not restricted to the MMP-inhibition, they also modulate other cellular processes.

Peer review

This is a study on matrix metalloproteinases and their tissue inhibitors in pouch mucosa of pediatric onset UC. This is a good study and the topic is so important because pouchitis is often seen in those who have undergone ileal pouch-anal anastomosis.

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BRIEF ARTICLE

Overexpression of the M2 isoform of pyruvate kinase is an adverse prognostic factor for signet ring cell gastric cancer

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Abstract

AIM: To investigate M2 isoform of pyruvate kinase (PKM2) expression in gastric cancers and evaluate its potential as a prognostic biomarker and an anticancer target.

METHODS: All tissue samples were derived from gastric cancer patients underwent curative gastrectomy as a primary treatment. Clinical and pathological information were obtained from the medical records. Gene expression microarray data from 60 cancer and 19 non-cancer gastric tissues were analyzed to evaluate the expression level of PKM2 mRNA. Tissue microarrays were constructed from 368 gastric cancer patients. Immunohistochemistry was used to measure PKM2 expression and PKM2 positivity of cancer was determined by proportion of PKM2-positive tumor cells and staining intensity. Association between PKM2 expression and the clinicopathological factors was evaluated and the correlation between PKM2 and cancer prognosis was evaluated.

RESULTS: PKM2 mRNA levels were increased more than 2-fold in primary gastric cancers compared to adjacent normal tissues from the same patients (log transformed expression level: 7.6 ± 0.65 *vs* 6.3 ± 0.51, $P < 0.001$). Moreover, differentiated type cancers had significantly higher PKM2 mRNA compared to undifferentiated type cancers (log transformed expression level: 7.8 ± 0.70 *vs* 6.7 ± 0.71, $P < 0.001$). PKM2 protein was mainly localized in the cytoplasm of primary cancer cells and detected in 144 of 368 (39.1%) human gastric cancer cases. PKM2 expression was not related with stage ($P = 0.811$), but strongly correlated with gastric cancer differentiation ($P < 0.001$). Differentiated type cancers expressed more PKM2 protein than did the undifferentiated ones. Well differentiated adenocarcinoma showed 63.6% PKM2-positive cells; in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells. Importantly, PKM2 expression was correlated with shorter overall survival ($P < 0.05$) independent of stage only in signet-ring cell cancers.

CONCLUSION: PKM2 expression might be an adverse prognostic factor for signet-ring cell carcinomas. Its function and potential as a prognostic marker should be further verified in gastric cancer.

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INTRODUCTION

Gastric cancer is the second leading cause of cancer-related deaths worldwide[1]. Although surgery is the standard curative treatment for gastric cancer, relapses occur in many patients even after adjuvant therapy. Gastric cancer patients with the same stage of the disease present different clinical courses and have different prognosis[2]. This heterogeneity of gastric cancer is present at the molecular level and has a genetic predisposition to it[3-6]. Therefore, it is important to identify new molecular markers to predict patients' outcomes and personalize treatments according to the individual biology of each cancer.

Cancer cells take up glucose at higher rates than do normal cells but produce energy mainly by glycolysis, rather than by mitochondrial oxidation of pyruvate[7]. This process, called aerobic glycolysis or the Warburg effect, is very important for tumor growth[8]. Glycolysis increases lactate production resulting in acidification of the extracellular environment, which is believed to facilitate cell invasion and metastasis[9]. The M2 isoform of pyruvate kinase (PKM2) was identified as a driver of aerobic glycolysis, and has been shown to be the isoform preferentially overexpressed in tumor cells[10]. Other isoenzymes of pyruvate kinase (pyruvate kinase type M1, pyruvate kinase type L, pyruvate kinase type R) are expressed depending upon the metabolic responsibilities of the various non-cancerous cells and tissues[10].

Several studies have shown that PKM2 is selectively stained in cancer cells in immunohistochemical assay[11,12]. It has been suggested that plasma PKM2 could be a valuable tumor marker for diagnosis or monitoring of lung, pancreas, kidney, breast, tongue, and gastrointestinal cancers[11-17]. However, little is known about the biological function of PKM2 in cancer and its potential as an anti-cancer target. Previous studies reported that PKM2 protein level was increased in both the tumors and plasma of gastric cancer patients[17], and that it positively correlated with cisplatin sensitivity in gastric cancer cell lines[18]. However, clinical and prognostic implications of PKM2 as a marker for gastric cancer are still unclear. Therefore, we decided to analyze whether PKM2 expression is correlated with cancer progression and prognosis in human gastric cancer patients.

MATERIALS AND METHODS

Gene expression microarray data analysis

The previously generated gene expression data from 60 gastric cancer patients is available in the NCBI's GEO public database (microarray data accession number, GSE13861)[2]. All patients underwent curative gastrectomy as a primary treatment in 2005 at Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Clinical and pathologic data were obtained by review of the Severance Hospital medical records. The gene expression data of 60 cancer and 19 non-cancer gastric tissues from 60 gastric cancer patients were analyzed. Class comparison using two sample t test (significance $P < 0.001$, 10 000 random permutation) identified gastric cancer specific genes.

Patients and tissues

We selected primary gastric adenocarcinoma patients who had undergone curative gastrectomy as the primary treatment between 1999 and 2007 at Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Patients were followed up more than 36 mo after surgery or presented recurrence or death within 36 mo after surgery. We obtained paraffin-embedded tissues and clinical data from patients. The demographic details of the cases analyzed are described in Table 1. Clinical and pathological information were obtained from the medical records. Tumors were staged according to the 7th edition of the American Joint Committee Guidelines on cancer staging issued in 2010. Tumor histology was classified as differentiated (well and moderately differentiated adenocarcinoma) and undifferentiated (poorly differentiated adenocarcinoma and signet ring cell carcinoma) type. The median follow-up duration was 70.6 mo (range: 3.6-144.6 mo). A total of 125 (34%) patients did not receive any adjuvant chemotherapy, and most of their cancers were classified as stage I . No radiation was given to any of the patients. The study was approved by the Investigational Review Board of Gangnam Severance Hospital.

Tissue microarray construction and immunohistochemistry

The paraffin-embedded tissue microarray blocks of gastric cancer tissue specimens obtained from 368 patients were used. Each block had a 3-mm core of gastric cancer tissue. Immunohistochemistry was performed on 4 μm-thick tissue microarray tissue sections an Enzyme-conjugated polymer backbone: Dextran (EnVision Detection kit, DAKO Cytomation, Glostrup, Denmark) according to the manufacturer's instructions after microwave-based antigen retrieval. Antibody to PMK2 1:500, Cell Signaling, Cambridge, MA, United States) was applied to the sections, which were incubated for 2 h at room temperature. The sections were incubated with secondary antibody (HRP-Rabbit/Mouse) for 15 min at room temperature, and developed using a Nova-RED substrate kit (VECTOR Laboratory, Burlingame,

Table 1 Correlation between the M2 isoform of pyruvate kinase expression and clinicopathologic characteristics of gastric cancer patients *n* (%)

Characteristics	Total (*n* = 368)	M2 isoform of pyruvate kinase expression		*P*
		Negative (*n* = 224)	Positive (*n* = 144)	
Median follow-up (70.6 mo)				
Relapse	143 (39.0)			
Death	138 (37.7)			
Adjuvantchemotherapy	243 (66.0)			
Age (yr)				0.027
≤ 60	230	150 (65.2)	80 (34.8)	
> 60	138	74 (53.6)	64 (46.4)	
Gender				0.263
Male	222	130 (58.6)	92 (41.4)	
Female	146	94 (64.4)	52 (35.6)	
AJCC 7th stage				0.811
Ⅰ	105	67 (63.8)	38 (36.2)	
Ⅱ	89	51 (57.3)	38 (42.7)	
Ⅲ	172	105 (61.0)	67 (39.0)	
Ⅳ	2	1 (50.0)	1 (50.0)	
T stage				0.009
T1	94	62 (65.9)	32 (34.1)	
T2	42	30 (71.4)	12 (28.6)	
T3	87	40 (45.9)	47 (54.1)	
T4	145	92 (63.4)	53 (36.6)	
N stage				0.086
N0	131	80 (61.1)	51 (38.9)	
N1	62	33 (53.2)	29 (46.8)	
N2	69	37 (53.6)	32 (46.4)	
N3	106	74 (69.8)	32 (30.2)	
Histology				< 0.001
Welldifferentiatedadenocarcinoma	22	8 (36.4)	14 (63.6)	
Moderatelydifferentiatedadenocarcinoma	96	39 (40.6)	57 (59.4)	
Poorlydifferentiatedadenocarcinoma	143	91 (63.6)	52 (36.4)	
Signetringcellcarcinoma	79	65 (82.3)	14 (17.7)	

AJCC: American Joint Committee on Cancer; T: Tumor; N: Node.

CA, United States) and counterstained with Harris hematoxylin. The slides were photographed using a Zeiss microscope. The degree of immunostaining was scored independently by 2 observers based on the proportion of positively stained tumor cells and the intensity of staining. Tumor cell proportion was classified as follows: 0%, 10%-25%, 25%-50%, and > 50% PKM2-positive tumor cells. Staining intensity was classified as none, weak and strong staining.

We measured PKM2 expression in non-cancer gastric epithelial cells and malignant lesions. Tumors with more than 25% PKM2-positive cells were considered tumors with positive PKM2 expression, and those with less than 25% PKM2-positive cells were considered negative for PKM2 expression.

Statistical analysis

The correlation between the immunohistochemical expression scores and patient survival after surgery was estimated using the Kaplan-Meier method, followed by univariate comparison between the groups using the log-rank test. To adjust for potential confounding variables and to single out independent predictors of survival, a multivariate analysis of survival was done using the Cox's proportional hazard model using a forward stepwise mode. Statistical analyses were done with GraphPad Prism 5 (GraphPad Software, San Diego, CA) and PASW Statistics 18.0 (SPSS Inc., Chicago, IL). Association between PKM2 expression and the clinicopathological factors was tested using the χ^2 test. Two-tailed *P* values of 0.05 or less were considered statistically significant.

RESULTS

Upregulation of PKM2 mRNA in primary gastric cancers

From sixty gastric cancer patients, 60 gastric cancer tissues and 19 non-cancer adjacent gastric tissues were used for gene expression microarray analysis. PKM2 was identified as one of 3360 gastric cancer-specific genes by class comparison using the 2-sample *t* test (Data not shown). PKM2 mRNA levels were increased > 2-fold in human primary gastric cancers compared to normal adjacent tissues from the same patients (log transformed expression level: 7.6 ± 0.65 *vs* 6.3 ± 0.51, *P* < 0.001, Figure 1A). Among cancer types, differentiated type cancers displayed > 2-fold increase in PKM2 levels compared to undifferentiated type cancers (log transformed expression level: 7.8 ± 0.70 *vs* 6.7 ± 0.71, *P* < 0.001, Figure 1B).

Overexpression of PKM2 in primary gastric cancer

To examine whether PKM2 protein upregulation was linked to the clinical characteristics of gastric cancers,
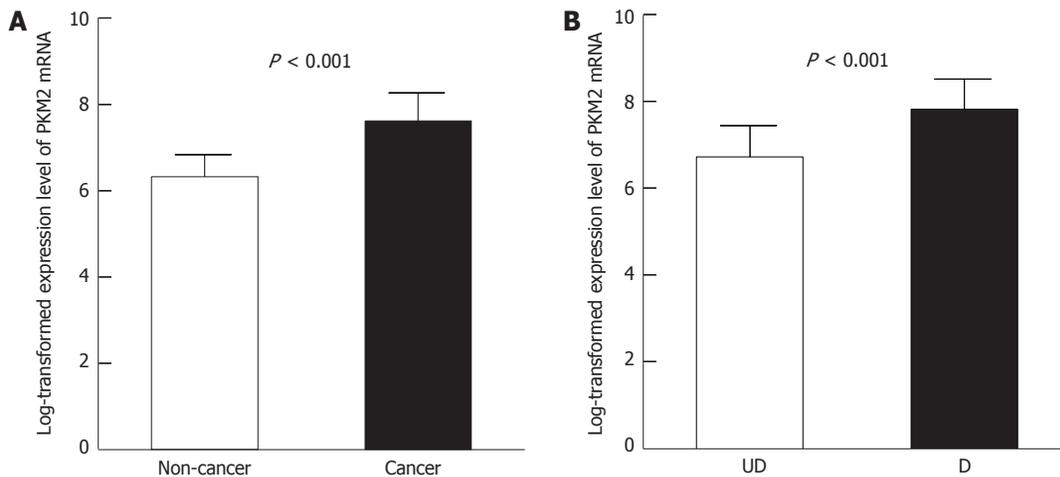
Figure 1 Expression level of the M2 isoform of pyruvate kinase mRNA by gene expression microarray. A: The M2 isoform of pyruvate kinase (PKM2) up-regulation in the 18 primary gastric cancers compared to gastric adjacent noncancerous tissues paired from the same patient ($P < 0.001$); B: PKM2 up-regulation in the 22 differentiated type (D) gastric cancers compared to 27 undifferentiated type (UD) gastric cancers ($P < 0.001$). The column and bar represent mean and standard deviation, respectively.
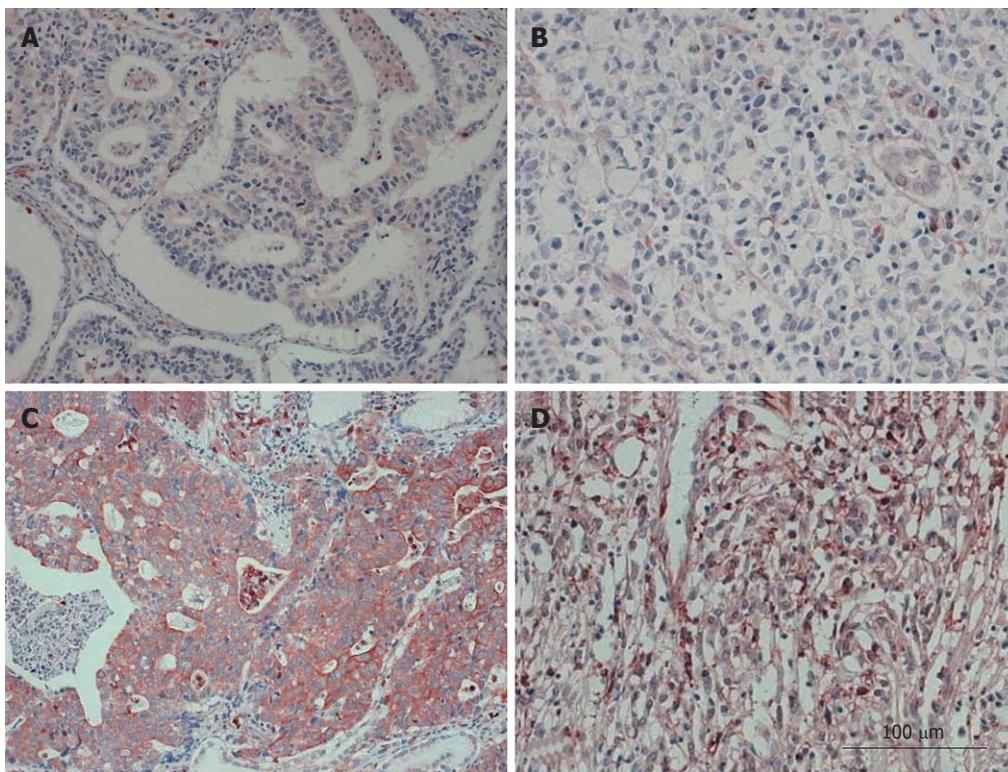
Figure 2 Representative images from immunohistochemistry assays of 368 archived gastric cancer cases at 20 × 10 magnification. A: Moderately differentiated adenocarcinoma and negative for the M2 isoform of pyruvate kinase (PKM2) expression; B: Signet ring cell carcinoma and negative for PKM2 expression; C: Moderately differentiated adenocarcinoma and positive for PKM2 expression; D: Signet ring cell carcinoma and positive for PKM2 expression.

the following samples were subjected to immunohistochemistry with a human PKM2 antibody: 368 paraffin-embedded, archived gastric cancer tissue samples, including 194 cases of stages I / II and 174 cases of stage III/IV tumors. The results are summarized in Table 1. PKM2 protein was detected in 144 of 368 (39.1%) human gastric cancer cases. Strong cytoplasmic staining of PKM2 was detected in 42 (11.4%) tumors and weak staining was detected in 102 (27.7%) tumors. As shown in Figure 2, PKM2 was mainly localized in the cytoplasm of primary cancer cells. Diffuse and/or intense cytoplasmic staining was noted in only cancer cells. In contrast, PKM2 was either undetectable or only marginally detectable in the normal epithelial body gland of noncancerous tissues in the adjacent section regions (Figure 2).

Relationship between PKM2 expression and the clinical features of gastric cancers

As shown in Table 1, there was no correlation between stage and PKM2 expression ($P = 0.811$). PKM2 expres-

Table 2 Prognosis analysis of recurrence-free survival and overall survival of total patients (*n* = 366)

Characteristics	RFS		OS	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (yr)				
⩽ 60				
> 60	1.12 (0.80-1.57)	0.488	1.16 (0.83-1.64)	0.373
Gender				
M				
F	1.18 (0.85-1.65)	0.315	1.09 (0.78-1.53)	0.600
PKM2				
Negative				
Positive	0.93 (0.66-1.32)	0.713	0.92 (0.65-1.30)	0.637
T stage				
T1/2/3				
T4	6.12 (4.25-8.81)	< 0.001	5.04 (3.51-7.22)	< 0.001
N stage				
N0/1/2				
N3	6.02 (4.29-8.46)	< 0.001	5.64 (4.01-7.95)	< 0.001
Stage				
I / II				
III	8.42 (5.48-12.94)	< 0.001	6.70 (4.41-10.16)	< 0.001

RFS: Recurrence-free survival; OS: Overall survival; HR: Hazard ratio; PKM2: The M2 isoform of pyruvate kinase; T: Tumor; N: Node; 95% CI: 95% confidence interval.

Table 3 Univariate and multivariate analysis of overall survival in signet ring cell carcinoma (*n* = 79)

Characteristics	Groups	HR (95% CI)	*P* value
Univariate analysis			
Age (yr)	> 60 *vs* ⩽ 60	1.11 (0.52-2.37)	0.785
Gender	F *vs* M	1.08 (0.56-2.07)	0.817
PKM2	Positive *vs* negative	2.13 (1.02-4.44)	0.042
T stage	T4 *vs* T1/2/3	6.25 (3.03-12.85)	< 0.001
N stage	N3 *vs* N0/1/2	5.70 (2.90-11.22)	< 0.001
Stage	III *vs* I / II	6.84 (2.83-16.53)	< 0.001
Multivariate analysis			
PKM2	Positive *vs* negative	2.12 (1.02-4.42)	0.044
Stage	III *vs* I / II	6.84 (2.83-16.55)	< 0.001

HR: Hazard ratio; PKM2: The M2 isoform of pyruvate kinase; 95% CI: 95% confidence interval; F: Female; M: Male; T: Tumor; N: Node.

sion was strongly correlated with gastric cancer differentiation (*P* < 0.001). Differentiated type cancers expressed more PKM2 protein than did the undifferentiated ones. Well differentiated adenocarcinoma showed 63.6% PKM2-positive cells; in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells.

Association between PKM2 expression and patient prognosis

We evaluated whether PKM2 expression could be a prognostic factor for gastric cancer. Two out of 368 patients died of non-cancer and were excluded from analysis. Table 2 shows that recurrence-free survival (RFS) and overall survival (OS) are significantly different between patients with different clinical stage (*P* < 0.001), T classification (*P* < 0.001), and N classification (*P* < 0.001). There was no significant difference in prognosis according to PKM2 expression.
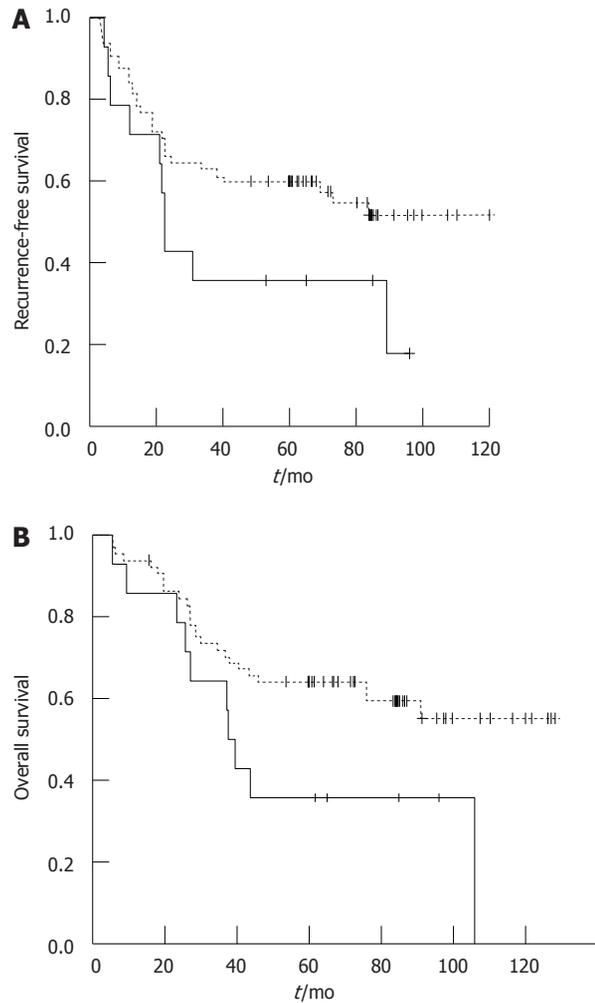
Figure 3 Recurrence-free survival (A) and overall survival (B) curves according to the M2 isoform of pyruvate kinase expression in signet ring cell gastric cancer after curative resection (*n* = 79). Positive M2 isoform of pyruvate kinase (PKM2) expression, real line; Negative PKM2 expression, dotted line.

We performed subgroup analysis at each tumor stage. In stages II or III patients, PKM2 expression showed no significant correlation with RFS or OS. However, in stage I early gastric cancer patients (*n* = 99), PKM2 expression was significantly correlated with poor RFS (*P* = 0.006) and OS (*P* = 0.015). Based on the observation that PKM2 expression rate was remarkably different according to cancer histology (Table 1), the prognostic value of PKM2 expression in patient subgroups was evaluated according to the histology. We found that in signet-ring cell carcinomas PKM2 expression correlated with poor prognosis (*P* = 0.042 for OS, Table 3 and Figure 3). Moreover, univariate and multivariate analyses showed that PKM2 expression, as well as clinical stage, were independent prognostic factors for survival (Table 3).

DISCUSSION

In this study, we report the characterization of PKM2 expression in human gastric cancers, and present its correlation with clinicopathological parameters and patients' prognosis. First, our study revealed that PKM2 is overex-

pressed in gastric cancers both at the mRNA and protein levels compared to normal gastric tissues. Well and moderately differentiated adenocarcinoma showed significantly higher expression of PKM2 (60% PKM2-positive cells); in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells (Table 1). Because PKM2 is mainly localized in the cytoplasm of primary cancer cells and signet-ring cells contain a large amount of mucin and scanty cytoplasm, we hypothesize that this might explain the lower levels of PKM2 expression in these cells. This finding might be explained by the different glucose utilization rates of the various gastric cancer subtypes. Fluorine-18 fluoro deoxy-D-glucose positron emission tomography detected glucose uptake of tumor cells, and differentiated gastric cancers showed higher fluoro deoxy-D-glucose uptake rates than did undifferentiated ones[19].

Second, PKM2 protein expression was found to negatively correlate with survival in signet-ring cell gastric cancer patients, as higher expression of PKM2 is associated with patients' shorter survival time ($P = 0.042$) after curative resection (Figure 3). Signet-ring cell carcinomas have a distinct biology and generally have worse prognosis than do other types of gastric cancer[20]. A recent study reported that higher glucose uptake was indicative of a more aggressive disease especially in advanced signet-ring cell cancers[21], although no biological mechanism was proposed to explain it. This finding is in agreement with our results. Thus, our study suggests that higher PKM2 expression, which indicates a higher rate of glycolysis in the tumor, might represent a novel prognostic marker for the clinical outcome of these types of gastric cancers.

PKM2 expression was related with poor prognosis only in stage I gastric cancer patients who did not receive chemotherapy. Only 4 of 99 patients showed relapse after curative gastrectomy, and in all cases, cancer cells were positive for PKM2 expression compared to the 36% patients overall who expressed PKM2. Furthermore, 3 patients had early relapses, within 1 year from the surgery, and all expressed high levels of PKM2 in the resected tissues. As cancer relapse in stage I patients are rare and four recurrent cases in our result are small number, it seems too early to conclude that PKM2 expression correlated with poor prognosis of stage I gastric cancer. However, it is clinical value to expand investigation in large cases. For stages II and III patients, there were no significant differences in survival. In a previous study, PKM2 was shown to positively correlate with the response to cisplatin in human gastric cancer cell lines[18]. Cisplatin is the main chemotherapeutic agent for gastric cancers as either adjuvant or palliative aim. As cisplatin was administered as adjuvant therapy to 62.8% (147/234) of stages II or III gastric cancer patients after curative gastrectomy, the negative prognostic effect of PKM2 might be cancelled by cisplatin-based chemotherapy.

The possibility of using PKM2 as a target for the development of anti-cancer therapies has been evaluated in the preclinical setting[22,23]. PKM2 knockdown by short hairpin RNA reduced the ability of human cancer cell lines to form tumors in nude mouse xenografts[10,24]. If anti-cancer strategies based on targeting PKM2 treatment are feasible, stage I or signet-ring cell cancer patients with PKM2 expression would be suitable candidates for such treatments.

The molecular function of PKM2 and its role in cancer are not completely understood yet. It was recently shown that PKM2 allows cancer cells to mount an antioxidant response and thereby support cell survival under acute oxidative stress[25] and also induces epidermal growth factor receptor (EGFR)-dependent β-catenin transactivation, which leads to cell proliferation and tumorigenesis[26]. These data are in agreement with our microarray study in which we also identified EGFR and β-catenin signaling, and hypoxic stress are linked to gastric cancer. Altogether, these studies suggest that the function of PKM2 in gastric cancer is very complex and needs to be further elucidated. In addition, the mechanisms of the regulation of PKM2 expression specifically in gastric tumors should be studied.

In conclusion, this study showed that PKM2 was overexpressed in gastric cancers. Moreover, PKM2 expression is an independent prognostic factor for signet ring cell carcinomas. The biological role of PKM2 in the development of these tumors needs to be further elucidated.

COMMENTS

Background
Gastric cancer is the major cause of cancer-related deaths worldwide. It is important to identify molecular markers to predict patients' outcomes and personalize treatments according to the individual biology. Clinical and prognostic implications of M2 isoform of pyruvate kinase (PKM2) as a marker for gastric cancer were unclear. The authors evaluated whether PKM2 expression is correlated with cancer progression and prognosis in human gastric cancer patients.

Research frontiers
PKM2 was identified as a driver of aerobic glycolysis, and has been shown to be overexpressed in tumor cells. PKM2 was selectively expressed in cancer cells and suggested valuable tumor marker for diagnosis or monitoring of various cancers. The biological function of PKM2 in cancer has been elucidated and PKM2 might be a candidate for anti-cancer target.

Innovations and breakthroughs
This study revealed that PKM2 was overexpressed in gastric cancers both at the mRNA and protein levels compared to normal gastric tissues and was found to negatively correlate with survival in signet-ring cell gastric cancer patients. PKM2 expression might be an adverse prognostic factor for signet-ring cell carcinomas. Its function and potential as a prognostic marker should be further verified in gastric cancer.

Applications
It is plausible to use PKM2 as an adverse prognostic marker of signet-ring cell cancer patients. If anti-cancer strategies based on targeting PKM2 treatment are feasible, signet-ring cell cancer patients with PKM2 expression would be suitable candidates for such treatments.

Terminology
Aerobic glycolysis: Many tumor cells have elevated rates of glucose uptake but reduced rates of oxidative phosphorylation. This persistence of high lactate production by tumors in the presence of oxygen was known as aerobic glycolysis. This metabolic switch may be required to support cell growth. High aerobic glycolysis by malignant tumors is utilized clinically to diagnose and monitor treatment responses of cancers and also to treat cancer using antagonist.

Peer review
This study investigated PKM2 expression in 368 gastric cancers and evaluated its potential as a prognostic biomarker based on relapse and survival data of patients. The results indicate that PKM2 positive expression could be used as an adverse prognostic marker in signet-ring cell gastric cancer.

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BRIEF ARTICLE

Effects of vagus nerve preservation and vagotomy on peptide YY and body weight after subtotal gastrectomy

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Abstract

AIM: To investigate the relationship between the function of vagus nerve and peptide YY$_{3-36}$ and ghrelin levels after subtotal gastrectomy.

METHODS: We enrolled a total of 16 patients who underwent subtotal gastrectomy due to gastric cancer. All surgeries were performed by a single skilled surgeon. We measured peptide YY$_{3-36}$, ghrelin, leptin, insulin, growth hormone levels, and body weight immediately before and one month after surgery.

RESULTS: Vagus nerve preservation group showed less body weight loss and less increase of peptide YY$_{3-36}$ compared with vagotomy group (-5.56 ± 2.24 kg *vs* -7.85 ± 1.57 kg, *P* = 0.037 and 0.06 ± 0.08 ng/mL *vs* 0.19 ± 0.12 ng/mL, *P* = 0.021, respectively). Moreover, patients with body weight loss of less than 10% exhibited reduced elevation of peptide YY$_{3-36}$ level, typically less than 20% [6 (66.7%) *vs* 0 (0.0%), *P* = 0.011, odd ratio = 3.333, 95% confidence interval (1.293, 8.591)].

CONCLUSION: Vagus nerve preservation contributes to the maintenance of body weight after gastrectomy, and this phenomenon may be related to the suppressed activity of peptide YY$_{3-36}$.

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INTRODUCTION

Body weight loss is a common and serious outcome in patients with gastric cancer who are treated by gastrectomy[1]. Weight loss is correlated with declines in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, infection susceptibility, and survival[2,3]. Postoperative body weight loss can be explained by mechanisms such as reduced food intake, appetite loss caused by the reduced reservoir or delayed gastric emptying[4,5], diarrhea[6] and malabsorp-

tion. Malabsorption, in turn, is linked to reduced secretion of gastric acid[7] and pancreatic insufficiency[8,9]. In addition, it was recently suggested that alterations of endocrine status, such as reduced gastrin[8] or ghrelin[10], and increased cholecystokinin levels[5,8] might be involved in weight loss after gastrectomy. However, the mechanism of body weight loss after gastrectomy has not been fully clarified.

The 28-amino acid peptide ghrelin is the endogenous ligand for the growth hormone secretagogue receptor 1a, which stimulates the release of growth hormone from the pituitary gland[11]. The majority of ghrelin is produced by X/A-like endocrine cells of the gastric oxyntic mucosa, and smaller amounts are secreted by other organs, such as the intestine, pancreas, kidney and hypothalamus[12,13]. Ghrelin has a number of physiologic effects that result in positive energy balance, such as promoting the appetite signal in the hypothalamus as an antagonist to leptin[14], stimulating gastrointestinal activities such as peristalsis, gastric acid secretion, and pancreatic excretion through the vagal nerves[15], and regulation of fat metabolism. Peptide YY3-36, a 36 amino acid gut-derived hormone, reduces food intake over the short term in animals[16,17] and in humans[18] by stimulating hypothalamic neuropeptide Y receptors. Preprandial decreases and postprandial increases in plasma peptide YY3-36 concentrations suggest that peptide YY3-36 is one of satiety signals. Peptide YY3-36 is suggested to be involved in intermediate term inhibition of food intake, in contrast to the classical short term regulators such as cholecystokinin[16]. Dose-dependent reductions in food intake following peripheral peptide YY3-36 administration are observed in both fasting and freely feeding rodents[16,17,19,20]. In healthy human volunteers, intravenous infusion of peptide YY3-36 caused a sustained decrease in appetite and food intake for more than 24 h[18]. Moreover, gastric bypass results in a more robust peptide YY3-36 response to caloric intake, which, in conjunction with decreased ghrelin levels, may contribute to the sustained efficacy of this procedure[21]. One animal study suggested that peptide YY release is inhibited through a vagal cholinergic mechanism due to significant elevations of basal and food-induced release of peptide YY after truncal vagotomy[22].

Recently, one study reported that reductions in visceral fat were significantly lower in patients in whom the vagus nerve was preserved than in patients who had undergone vagotomy, and concluded that the vagus nerve locally regulates amounts of intra-abdominal fat tissue[23]. However, they did not mention the hormonal changes regarding the effect of vagotomy and vagus nerve preservation on body weight loss. Therefore, we aimed to reveal the correlation between the effect of vagus nerve preservation and vagotomy on peptide YY3-36 or ghrelin levels after subtotal gastrectomy in relation to body weight loss.

MATERIALS AND METHODS

Patients
Sixteen patients who underwent subtotal gastrectomy at Gospel Hospital, Kosin University College of Medicine, Busan, South Korea between January 2008 and January 2010 were enrolled in the study. The inclusion criteria were as follows: (1) adenocarcinoma of the stomach confirmed by histopathologic examination; (2) preoperative clinical staging of less than stage ⅢA (International Union Against Cancer tumor, node, metastasis stage classification); (3) curative surgical treatment (R0) (i.e., subtotal gastrectomy with D1 or D2 lymph node dissection); and (4) age between 20 and 80 years. The exclusion criteria were the presence of any of the following: (1) cardiopulmonary, liver, or renal dysfunction; (2) active dual malignancy; (3) pregnancy; (4) past history of gastrointestinal surgery; and (5) postoperative complications after subtotal gastrectomy that could affect oral food intake, such as anastomotic leakage, pancreatitis, and mechanical ileus. Sixteen patients were randomized by sealed-envelope selection and divided into two study groups. The random allocation sequence was concealed until interventions were assigned. Nine patients were treated by subtotal gastrectomy with vagus nerve preservation (vagus nerve preservation group), and seven patients underwent both subtotal gastrectomy and vagotomy (vagotomy group). The study was approved by the Kosin University Ethics Committee, and all patients provided written informed consent before study entry in accordance with the Declaration of Helsinki.

Operative procedures
In seven cases, subtotal gastrectomy and bilateral truncal vagotomy were performed with D1 or D2 lymph node dissection followed by Billroth-I or Roux-en-Y reconstruction. The hepatic and celiac branches of the vagus nerve were preserved in nine patients who underwent Billroth-I or Roux-en-Y reconstruction after subtotal gastrectomy with D1 or D2 lymph node dissection. The greater omentum was largely preserved in all cases. All operations were performed by a surgeon with a history of over 1000 gastric cancer operations over the course of 20 years. An ultrasonic knife (UltracisionÂ®, Ethicon Endo Surgery, Cincinnati, OH, United States) was used to prevent nerve damage in the vagus nerve preservation group. Electrical impulses were produced by a high-frequency ultrasound generator, transferred to a hand piece and converted into mechanical movement at a frequency of 55.5 kHz. This instrument was chosen because of the relatively low temperatures generated, ranging from 50 ℃ to 100 ℃, which limit damage to adjacent tissue compared to conventional diathermy. Diathermy produces temperatures up to 400 ℃, resulting in char formation and deleterious thermal effects to a distance of up to 1 cm from the blade, as well as the extensive formation of necrotic tissue.

Body weight and blood sampling for hormone measurement in the fasted state
Peptide YY3-36, ghrelin, leptin, insulin, and growth hormone levels as well as body weights were measured 1 d before surgery (day 1) and 1 mo after surgery (day 30). Venous fasting blood samples were taken early in the

Total 16 patients with AGC from January 2008 to January 2010

Body weight check, hormone assay and randomization at POD-1

Nine cases STG with vagus nerve preservation

Seven cases STG with vagotomy

Body weight check and hormone assay at POD 30
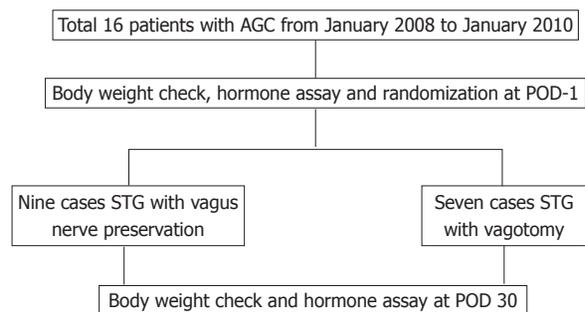
Figure 1 Flowchart for investigating effects of vagus nerve preservation and vagotomy on body weight and hormones such as peptide YY, ghrelin, leptin, insulin and growth hormone after total gastrectomy due to gastric cancer. AGC: Advanced gastric cancer; POD: Postoperative day; STG: Subtotal gastrectomy.

morning on day 1 and day 30 for the measurement of plasma concentrations of the following hormones: peptide YY_{3-36} (enzyme-linked immunosorbent assay, Phoenix Pharmaceuticals Inc., Belmont, CA, United States), total ghrelin (radioimmunoassay, Linco Research Inc., St. Louis, MO, United States), leptin (radioimmunoassay, Linco Research Inc.), insulin (two-site sandwich immunoassay, Siemens Medical Solutions Diagnostics, Los Angeles, CA, United States), and human growth hormone (radioimmunometric assay, Packard Instruments Inc., Chicago, IL, United States). Peptide YY_{3-36}, insulin, and growth hormone levels were measured with the V-MAX 220 VAC enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, United States), ADVIA Centaur XP (Siemens, Tarry-town, NY, United States), and COBRA II Gamma counter (PacKard, Waltham, MA, United States), respectively. Ghrelin and leptin were analyzed with the COBRA II Gamma Counter (PacKard, United States). Total protein, albumin, total cholesterol, and triglyceride levels were tested with the ADVIA 2400 (Siemens, Tarry-town, NY, United States).

Statistical analysis

Statistical analysis was performed using the SPSS software (version 16.0, SPSS, Chicago, IL, United States). Differences between the vagus nerve preservation group and the vagotomy group, including sex, age, stages of gastric cancer, body weight, body mass index (BMI), and laboratory values were assessed by Fisher's exact tests and Mann-Whitney U test. Wilcoxon signed rank test was used to calculate the changes between pre and post-operative values in body weight, BMI, peptide YY_{3-36}, ghrelin, leptin, insulin, growth hormone, and other laboratory profiles of all patients. The differences in body weight, BMI, hormones, and other laboratory values between two groups one month after operation were calculated by Mann-Whitney U test. Fisher's exact tests were used to validate the correlation among vagus nerve preservation, body weight loss of less than 10%, and peptide YY_{3-36} level increases of less than 20 %. Statistical significance was set at a P value of < 0.05.

Table 1 Baseline characteristics of patients in two groups

Operative procedure	Vagus nerve preservation ($n = 9$)	Vagotomy ($n = 7$)	P
Age (yr)	59.01 ± 13.12	52.74 ± 6.40	NS
Sex (male/female)	6/3	4/3	NS
Body weight (kg)	62.01 ± 11.22	63.13 ± 6.01	NS
BMI (kg/m^2)	23.21 ± 2.40	24.41 ± 3.53	NS
TNM stage			NS
II	5 (55.5)	3 (42.8)	
IIIa	4 (45.5)	4 (57.2)	
Pathologic type			NS
Differentiated	6 (66.6)	4 (57.2)	
Undifferentiated	3 (33.4)	3 (42.8)	
Reconstruction			NS
Billroth-1	5 (55.5)	5 (71.4)	
Roux-en-Y	4 (45.5)	2 (28.6)	
Laboratory values			
Peptide YY_{3-36} (ng/mL)	0.38 ± 0.10	0.42 ± 0.07	NS
Ghrelin (pg/mL)	693.71 ± 211.25	703.90 ± 238.89	NS
Leptin (ng/mL)	3.88 ± 4.59	5.50 ± 7.99	NS
Insulin (mIU/L)	6.36 ± 3.95	6.47 ± 4.66	NS
Growth hormone (ng/mL)	1.51 ± 2.34	1.4 ± 0.18	NS
HOMA index	1.70 ± 1.24	1.68 ± 1.38	NS
Albumin (g/dL)	3.71 ± 0.43	3.63 ± 0.52	NS
Total protein (g/dL)	6.93 ± 0.50	6.92 ± 1.04	NS
Total cholesterol (mg/dL)	204.91 ± 25.23	209.63 ± 21.11	NS
Triglyceride (mg/dL)	171.72 ± 49.21	140.41 ± 45.24	NS

Data were expressed as mean ± SD or n (%). BMI: Body mass index; TNM: Tumor, node, metastasis; HOMA: Homeostatic model assessment; NS: Not significant.

RESULTS

Patient characteristics

The study flow diagram is summarized in Figure 1. There was no significant difference in age, sex, body weight, BMI, clinical stage of gastric cancer, pathologic types, and laboratory profiles including hormone values between the two groups. Table 1 summarizes the clinical and laboratory background of the 16 patients who completed the study.

Changes of body weight and hormones

All patients demonstrated body weight loss (preoperative body weight: 62.51 ± 9.02 kg *vs* postoperative body weight: 56.02 ± 8.32 kg, $P < 0.001$) with decreased BMI (23.71 ± 2.94 kg/m^2 *vs* 21.33 ± 2.62 kg/m^2, $P < 0.001$). All patients have increased peptide YY_{3-36} (0.41 ± 0.09 ng/mL *vs* 0.52 ± 0.15 ng/mL, $P = 0.020$), and decreased ghrelin (787.34 ± 421.33 pg/mL *vs* 506.21 ± 201.10 pg/mL, $P = 0.007$) postoperatively. Insulin levels were significantly increased in most patients (4.59 ± 6.12 mIU/L *vs* 1.86 ± 1.49 mIU/L, $P = 0.001$). There was no correlation between the basal values of peptide YY_{3-36} and the extent of body weight loss. There were no significant changes in leptin and growth hormone levels after surgery. No significant differences were found in albumin, protein, triglyceride, and total cholesterol levels either. Vagus never preservation group showed less decrease in

Table 2 Changes of body weight, body mass index and laboratory values between pre-operation and one month after operation and parameters at 1 mo after operation in two groups

Operative procedure	Changes from pre-operation to one month after operation			One month after operation		
	Vagus nerve preservation (*n* = 9)	Vagotomy (*n* = 7)	*P*	Vagus nerve preservation (*n* = 9)	Vagotomy (*n* = 7)	*P*
Body weight (kg)	-5.56 ± 2.24	-7.85 ± 1.57	0.037[1]	56.44 ± 10.40	55.29 ± 4.99	NS
BMI (kg/m^2)	-1.91 ± 1.04	-2.91 ± 0.39	0.031[2]	21.21 ± 2.08	21.44 ± 3.39	NS
Peptide YY$_{3-36}$ (ng/mL)	0.06 ± 0.08	0.19 ± 0.12	0.021[3]	0.44 ± 0.07	0.62 ± 0.17	0.020[4]
Ghrelin (pg/mL)	-229.23 ± 196.69	-174.47 ± 174.58	NS	464.47 ± 235.49	529.43 ± 134.86	NS
Leptin (ng/mL)	-2.08 ± 3.38	-3.56 ± 7.41	NS	1.79 ± 1.31	1.94 ± 1.79	NS
Insulin (mIU/L)	9.5 ± 12.37	11.01 ± 8.26	NS	15.86 ± 10.89	17.48 ± 7.48	NS
HOMA index	0.33 ± 0.15	0.35 ± 0.43	NS	2.10 ± 1.79	2.25 ± 1.99	NS
Growth hormone (ng/mL)	0.07 ± 3.32	0.19 ± 0.12	NS	1.58 ± 2.20	1.95 ± 2.44	NS
Albumin (g/dL)	-0.05 ± 0.32	-0.14 ± 0.66	NS	3.64 ± 0.31	3.50 ± 0.43	NS
Total protein (g/dL)	-0.17 ± 0.71	-0.18 ± 0.83	NS	6.77 ± 0.41	6.78 ± 0.88	NS
Total cholesterol (mg/dL)	-4.00 ± 15.21	-6.57 ± 10.42	NS	200.88 ± 22.31	203.00 ± 27.82	NS
Triglyceride (mg/dL)	-14.33 ± 20.40	-11.71 ± 18.93	NS	157.44 ± 38.29	128.71 ± 36.66	NS

All data were expressed as mean ± SD. [1]95% confidence interval (CI) = -4.445 to -0.157; [2]95% CI = -1.902 to -0.103; [3]95% CI = -0.224 to -0.022; [4]95% CI = -0.320 to -0.032. BMI: Body mass index; HOMA: Homeostatic model assessment; NS: Not significant.

body weight (-5.56 ± 2.24 kg *vs* -7.85 ± 1.57 kg, *P* = 0.037) and BMI (-1.91 ± 1.04 kg/m^2 *vs* -2.91 ± 0.39 kg/m^2, *P* = 0.031) than vagotomy group. Moreover, less elevation of peptide YY$_{3-36}$ was observed in vagus nerve preservation group (0.06 ± 0.08 ng/mL *vs* 0.19 ± 0.12 ng/mL, *P* = 0.021) than vagotomy group. Total protein, albumin, total cholesterol, and triglyceride levels did not show significant changes between two groups after surgery. These results were presented in Table 2. Vagus never preservation group showed significantly lower post-operative peptide YY$_{3-36}$ value than vagus nerve preserved group (0.44 ± 0.07 ng/mL *vs* 0.62 ± 0.17 ng/mL, *P* = 0.020). However, there were no differences in other post-operative values between two groups. These post-operative findings were described in Table 2.

Relationships among vagus nerve preservation, body weight change, and peptide YY$_{3-36}$ change

Patients were separated into groups based on the changes of body weight and peptide YY$_{3-36}$. Body weight loss of less than 10% was more frequently observed in vagus nerve preservation group [6 (66.7%) *vs* 0 (0.0%), *P* = 0.011, odd ratio (OR) = 3.333, 95% confidence interval (95% CI) (1.293, 8.591); Figure 2A]. Increases of peptide YY$_{3-36}$ less than 20% was more frequently noticed in the vagus nerve preservation group after surgery [5 (55.6%) *vs* 0 (0.0%), *P* = 0.034, OR = 2.750, 95% CI (1.258, 6.010); Figure 2B]. Patients with body weight loss of less than 10% exhibited significant correlation with reduced elevation of peptide YY$_{3-36}$ level, typically less than 20% [5 (83.3%) *vs* 0 (0.0%), *P* = 0.001, OR = 11.000, 95% CI (1.697, 71.282); Figure 2C].

DISCUSSION

Weight loss is a common complication after gastrectomy. However, the effects of different surgical procedures associated with gastrectomy on postoperative body weight loss and hormonal changes are not well understood. In the present study, we examined changes in body weight, peptide YY$_{3-36}$, ghrelin, leptin, growth hormone, and insulin levels in vagus nerve preservation and vagotomy groups.

Although all 16 patients who underwent subtotal gastrectomy exhibited body weight loss, the vagus nerve preservation group demonstrated significantly less decreases in body weight and BMI than the vagotomy group. This study reproduces the findings of Melissas *et al*[7] who postulated that this phenomenon might be explained by the energy saving function of the vagus nerve as a major parasympathetic nerve innervating visceral organs. The sympathetic nervous system predominates during energy-spending catabolic states, whereas in energy-saving anabolic states the parasympathetic nervous system prevails[24,25]. Anatomical and physiological studies have demonstrated the innervation of adipose tissue by sympathetic nerves, which in turn accelerate lypolysis of adipocytes[26,27]. The vagotomy group showed significantly greater visceral fat mass reduction than the vagus nerve preservation group[7]. In our study, vagus nerve preservation presented 3.333 fold more chance than vagotomy group for body weight loss less than 10% (95% CI from 1.293 to 8.591).

All patients in this study showed increased peptide YY$_{3-36}$ levels after subtotal gastrectomy. A previous study demonstrated that stomach gastrin inhibited peptide YY secretion in rats[28]. All study patients lost the antrum after subtotal gastrectomy, therefore reducing the effect of stomach gastrin on peptide YY. This is a possible explanation for the elevation of peptide YY levels in these patients. An alternative explanation for this phenomenon is that there is a negative correlation between fasting peptide YY$_{3-36}$ and markers of adiposity[18,29-31]. In addition, fasting peptide YY levels are significantly higher in anorexia nervosa sufferers than normal weight controls. In rodent studies, mice exposed to a high-fat diet develop
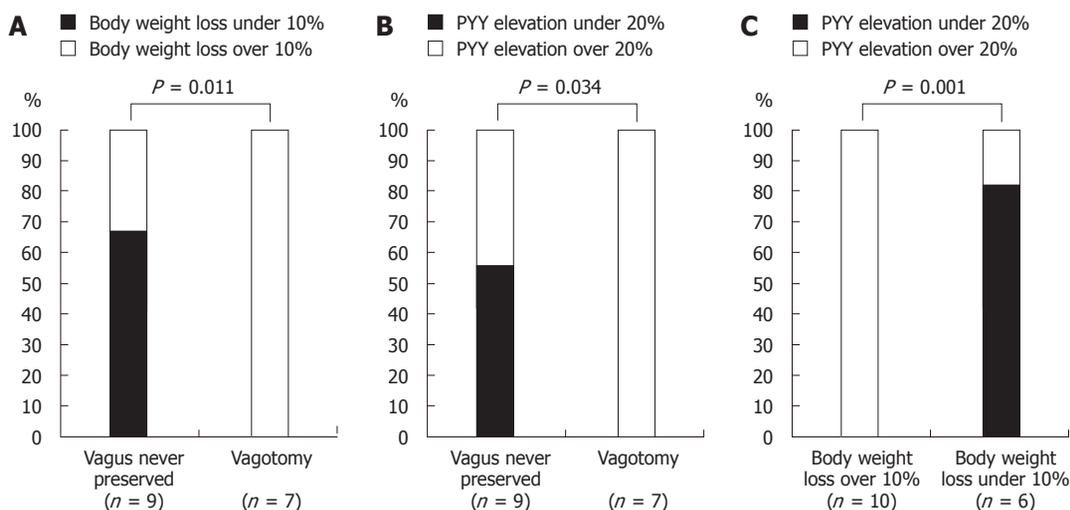
Figure 2 Correlations among vagus nerve preservation, body weight loss and peptide YY$_{3-36}$ increase. A: Vagus nerve preservation was correlated with body weight loss less than 10% [66.7% *vs* 0.0%, *P* = 0.011, odd ratio (OR) = 3.333, 95% confidence interval (95% CI) (1.293, 8.591)]; B: The vagus nerve preservation group showed significant correlation with peptide YY$_{3-36}$ level increases of less than 20% [55.6% *vs* 0.0%, *P* = 0.034, OR = 2.750, 95% CI (1.258, 6.010)]; C: Patients with body weight loss of less than 10% exhibited tight correlation with reduced elevation of peptide YY$_{3-36}$ level, typically less than 20% [83.3% *vs* 0.0%, *P* = 0.001, OR = 11.000, 95% CI (1.697, 71.282)]. PYY: Peptide YY.

obesity and a concomitant reduction in circulating peptide YY[32,33].

Vagus nerve preservation group demonstrated significantly less increase in peptide YY$_{3-36}$ than vagotomy group in the present study. Moreover, the vagus nerve preservation group demonstrated a tight correlation with increases in peptide YY$_{3-36}$ levels of less than 20%. According to a previous animal study, truncal vagotomy resulted in significant elevations of basal and food-induced release of peptide YY[22]. This suggests that peptide YY release is inhibited tonically, probably through a vagal cholinergic mechanism. Adrenergic pathways did not participate in food-stimulated peptide YY release. However, electrical stimulation of the splanchnic nerves increased basal levels of peptide YY, suggesting that the sympathetic nervous system affects the release of peptide YY[22]. This agrees with our finding that the vagus nerve preservation group showed reduced elevation of peptide YY$_{3-36}$ levels in comparison to the vagotomy group, although increased peptide YY$_{3-36}$ levels were observed in both groups. An alternative explanation for more elevation of peptide YY$_{3-36}$ in the vagotomy group could be a compensatory increase in peptide YY$_{3-36}$ secretion in response to reduced peptide YY$_{3-36}$ signaling to the hindbrain *via* the vagus. Thus peptide YY$_{3-36}$ may exert its effects on body weight by acting centrally, *via* vagal stimulation, or both. Many lines of evidence suggest that peptide YY$_{3-36}$ exerts its effects on feeding *via* the hypothalamus; intra-arcuate injection of peptide YY$_{3-36}$ reduces feeding, whereas Y2-antagonist injection has the opposite effect[34]. Thus, vagotomy, transection of hindbrain-hypothalamic pathways, can cause compensatory increase of peripheral peptide YY$_{3-36}$.

When correlations between body weight loss and increase of peptide YY$_{3-36}$ levels were performed, patients who demonstrated body weight loss of less than 10% ex-

hibited lower increases in peptide YY$_{3-36}$ levels, less than 20%. Because vagus nerve preservation is associated with lower increases in peptide YY$_{3-36}$ levels and reductions in body weight loss, it is not clear whether the lower increase in peptide YY$_{3-36}$ levels actually caused less body weight loss. However, we cannot exclude the possibility that peptide YY$_{3-36}$ levels influence body weight, as peptide YY$_{3-36}$ has been shown to suppress appetite and promote weight loss[16,17,19,20,35]. In healthy human volunteers, intravenous infusion of peptide YY$_{3-36}$ causes sustained decreases in appetite and food intake for more than 24 h[18]. Energy intake by obese subjects during a buffet lunch was reduced by 30% after intravenous infusion of peptide YY$_{3-36}$[18]. Chronic administration of peptide YY$_{3-36}$ inhibits food intake and reduced body weight gain in mice, rabbits, and rhesus macaques[16,35,36]. In addition, daily food intake, body weight, and body fat are increased in peptide YY knockout mice in comparison to wild-type mice[32]. Although the number of patients enrolled in the present study was limited, these findings suggest that elevated basal peptide YY$_{3-36}$ levels may contribute to body weight loss after subtotal gastrectomy.

All patients in this present study demonstrated increased plasma insulin after operation. A previous study showed the partial gastrectomy and intestinal resection induced impaired oral glucose tolerance despite normal insulin concentrations[37]. Increased basal level insulin might reflect the impaired insulin tolerance in the present study although it was not proved by oral glucose tolerance test.

There are five limitations in our study. First, patient appetite was not assessed. Assessing subjects' appetite on a visual analogue scale before and after surgery would have allowed us to evaluate the relationship between changes in peptide YY$_{3-36}$ levels and changes in appetite. Second, unfortunately, this present study did not include

data from meal-stimulated secretions of peptide YY or other hormones such as glucagon like peptide-1, which could have shed more light on the true interaction effects between vagus nerve preservation/vagotomy and gastrointestinal hormonal functions and body weight. Third, we did not evaluate body composition. Evaluating changes in body composition may have helped to elucidate the correlations between vagus nerve preservation, changes in peptide YY_{3-36} levels, and changes in specific body composition, especially visceral fat levels. Fourth, only total ghrelin was measured, since active octanoylated ghrelin is unstable. Although both total and active ghrelin appear to be regulated in a similar and parallel manner, future studies will need to focus on measurement of the biologically active form. Finally, small number of patients was enrolled in the present study.

In summary, body weight loss, increased peptide YY_{3-36} levels, and decreased ghrelin levels were observed in all patients after subtotal gastrectomy. Vagus nerve preservation group showed less decrease in body weight and BMI than vagotomy group. Less increase of peptide YY_{3-36} was observed in vagus nerve preservation group. Moreover, patients with body weight loss of less than 10% exhibited reduced elevation of peptide YY_{3-36} level, typically less than 20%. Based on these results and those of previous studies, we concluded that vagus nerve preservation resulted in reduced body weight loss after subtotal gastrectomy, in direct relation with peptide YY_{3-36} activities and suggest that vagus nerve should be preserved for preventing excessive body weight loss after subtotal gastrectomy due to gastric cancer.

COMMENTS

Background
Body weight loss is a common and serious outcome in patients with gastric cancer who are treated by gastrectomy. Weight loss is correlated with declines in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, infection susceptibility, and survival. Patients who underwent vagus nerve-preserving procedures lose less body weight than patients treated with vagotomy after gastrectomy.

Research frontiers
Ghrelin has a number of physiologic effects that result in positive energy balance, such as promoting the appetite signal in the hypothalamus as an antagonist to leptin. Peptide YY_{3-36} is suggested to be involved in intermediate term inhibition of food intake, in contrast to the classical short term regulators such as cholecystokinin. Recently, one study reported that reductions in visceral fat were significantly lower in patients in whom the vagus nerve was preserved than in patients who had undergone vagotomy, and concluded that the vagus nerve locally regulates amounts of intra-abdominal fat tissue.

Innovations and breakthroughs
This study is the first to evaluate relationship between the differences in weight loss between patients treated with vagus nerve-preserving procedures and vagotomy and the changes of peptide YY_{3-36} and ghrelin levels after subtotal gastrectomy. Vagus nerve preservation group showed less decrease in body weight and BMI than vagotomy group. Less increase of peptide YY_{3-36} was observed in vagus nerve preservation group. Moreover, patients with body weight loss of less than 10% exhibited reduced elevation of peptide YY_{3-36} level, typically less than 20%. Based on these results, the authors concluded that vagus nerve preservation resulted in reduced body weight loss after subtotal gastrectomy, in direct relation with peptide YY_{3-36} activities

Applications
Present study showed that vagus nerve preservation resulted in less decrease

in body weight and BMI than vagotomy group. Furthermore, this study suggested plausible peptide YY_{3-36} activities in this phenomenon. Considering these findings, the authors cautiously suggest to preserve vagus nerve during subtotal gastrectomy for less body weight loss.

Peer review
This is a good experiment study in which authors analyze the cause of the differences in weight loss between patients treated with vagus nerve-preserving procedures and vagotomy in the view of the changes of peptide YY_{3-36} and ghrelin levels after subtotal gastrectomy. The findings that vagus nerve preservation resulted in reduced body weight loss after subtotal gastrectomy, in direct relation with peptide YY_{3-36} activities suggesting the possible role of peptide YY_{3-36} in this phenomenon.

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BRIEF ARTICLE

Human papilloma virus 16 E6 oncoprotein associated with p53 inactivation in colorectal cancer

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Abstract

AIM: To investigate the association between human papilloma virus (HPV) infection and colorectal cancer.

METHODS: Sixty-nine patients with pathologically confirmed primary colorectal cancer including 6 stage Ⅰ, 24 stage Ⅱ, 21 stage Ⅲ, and 18 stage Ⅳ patients were enrolled in this study to investigate whether HPV 16 could be involved in colorectal tumorigenesis. Nested-polymerase chain reaction (nested-PCR) was used to detect HPV16 DNA in colorectal tumor tissues and further confirmed by *in situ* hybridization (ISH). In addition, immunohistochemistry analysis was performed to examine the E6 oncoprotein in colorectal tumors. To verify whether E6 could inactivate the p53 transcriptional function, the levels of p21 and Mdm2 mRNA expression were evaluated by real-time reverse transcription (RT)-PCR.

RESULTS: Of the 69 colorectal tumors, HPV16 DNA was detected in 11 (16%) by nested-PCR, and HPV16 DNA was present in 8 of the 11 (73%) tumors which was confirmed by ISH. The presence of HPV16 DNA in colorectal tumors was not associated with patients' clinical parameters including age, gender, smoking status, tumor site; however, HPV16 infection was more common in stage Ⅰ patients than in late-stages patients (Ⅱ, Ⅲ and Ⅳ). We next asked whether HPV16 infection could be linked with colorectal cancer development. Immunohistochemical data indicated that 8 of the 11 HPV16 DNA-positive tumors had E6 oncoprotein expression. Moreover, we also observed that the adjacent normal tissues including endothelial cells, lymphocytes, fibroblasts, and gland cells in E6-positive tumors had E6 oncoprotein expression. In addition, 3 of the 4 (75%) E6-positive tumors carrying p53 wild-type had negative immunostaining, but one tumor had less p53 immunostaining. We further examined whether E6-positive and/or p53 mutated tumors reduce p53 transcriptional activity. Real-time RT-PCR analysis indicated that p21 and mdm2 mRNA expression levels in E6/p53-wildtype tumors were significantly lower than in their adjacent

normal tissues; as expected, E6-positive/p53-mutated tumors had lower p21 and mdm2 mRNA expression levels compared with their adjacent normal tissues. These results clearly indicate that the E6 oncoprotein expressed in p53 wildtype tumors may reduce p21 and mdm2 expression *via* p53 inactivation.

CONCLUSION: These results suggest that HPV16 infection may be involved in a subset of colorectal cancer, and we suggest that the transmission of HPV to the colon and rectum might occur through peripheral blood lymphocytes.

INTRODUCTION

A high risk of human papilloma virus (HPV) 16/18 infection has been documented to be involved in the development of cervical and anal genital cancers[1]. Among non-genital cancers, the association of HPV16/18 infection and oropharyngeal cancer was recently evidenced[2-4]. However, other non-genital cancers, including lung, breast, and colorectal cancers have not yet been identified[5-12]. This is due to a lack of clarity as to how HPV transmits to internal organs even though blood circulation has been suggested as a possible route of infection[13,14].

In the past two decades, a large body of research has demonstrated an association between HPV infection and colorectal cancer[5,6,15-17]. Studies have shown HPV16 to be the major HPV subtype in colorectal tumors[16,17]. However, inconsistent conclusions have been drawn on this issue because only HPV DNA detection is used to associate HPV and colorectal cancer; there is no evidence to demonstrate that HPV is involved in colorectal cancer development. It is well known that HPV DNA integration into the host chromosome plays a crucial role in HPV-associated tumorigenesis[18]. When HPV DNA was spliced at E2, E6 and E7 oncoproteins were expressed which inactivated the p53 and Rb pathways[18]. Therefore, in the present study, 69 tumors resected from colorectal cancer patients were enrolled to determine the presence

of HPV16 DNA by nested polymerase chain reaction (PCR) and *in situ* hybridization (ISH), and expressions of E6 and p53 proteins were evaluated by immunohistochemistry (IHC) in colorectal tumor paraffin serial sections. We explored whether HPV16 DNA could exist and express E6 oncoprotein to inactivate the p53 pathway in colorectal tumors. We next asked whether HPV16 DNA and E6 oncoprotein could be expressed in adjacent normal tissue cells, such as endothelial cells and lymphocytes, to understand whether colorectal tumors infected with HPV16 could be spread through blood circulation.

MATERIALS AND METHODS

Study subjects

We collected tumor specimens from 69 patients with colorectal cancer. All of these patients, including 34 females and 35 males who were admitted to the Department of Surgery at Chung Shan Medical University Hospital, Taichung, Taiwan between 2000 and 2005, were asked to submit a written informed consent approved by the Institutional Review Board. A series of examinations for pathological stages were conducted for each case by board-certified pathologists based on the criteria in the 7th edition of the American Joint Committee on Cancer. We collected information pertaining to personal characteristics from hospital reports. Smokers were defined as those who were active smokers or previous smokers and nonsmokers were those who had never smoked.

Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μm. All sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in phosphate-buffered saline (pH 7.2), the buffer that was used for all subsequent washes. For HPV16 E6 and p53 detection, sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0), and then incubated with a monoclonal anti-human p53 antibody (DAKO, DO7, Denmark; at a dilution of 1:250) for 60 min at 25 ℃ or with monoclonal anti-HPV16 (Santa Cruz, CA, United States) for 90 min at 25 ℃. The conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Copenhagen, Denmark) was performed to develop signals and the cells were counter-stained with hematoxylin. Negative controls were obtained by leaving out the primary antibody. The intensities of signals were evaluated independently by three observers. The results were evaluated independently by three observers and scored for the percentage of positive nuclei: score 0, no positive staining; score +, from 1% to 10%; score ++, from 1% to 50%; score +++, more than 50% positive cells. Positive control slides for p53 protein detection were purchased from DAKO (Denmark) and the cervical cancer tumor tissues with HPV16 were used as a positive control for HPV16 E6. The antibody dilution buffer replaced the antibodies to serve as a negative control.

Direct sequencing

Mutations in exons 5-8 of the *p53* gene were determined by direct sequencing of PCR products amplified from the DNA of tumor cells isolated by microdissection of the colorectal tumor tissues. DNA lysis buffer was used to lyse cells and then the solution was subjected to proteinase K digestion and phenol-chloroform extraction. Finally, the DNA was precipitated by ethanol. Target sequences were amplified in a 50 μL reaction mixture containing 20 pmol of each primer, 2.5 units of Taq polymerase (TAKARA Shuzo, Shiga, Japan), 0.5 mmol/L dNTPs, 5 μL PCR reaction buffer, and 1 μL genomic DNA as the template. Genomic DNA sequences extracted from the frozen sections were not adequate for an amplification of long fragment DNA sequences, and therefore, PCR products ranging from 200 to 400 bp were amplified for *p53* mutation analysis. Primers for β-actin, which act as an internal control, were included in each amplification reaction. The primers used in the reactions were E5S (5'TGCCCTGACTTTCAACTCTG3') and E5AS (5'GCTGCTCACCATCGCTATC3') for exon 5, E6S (5'CTGATTCCTCACTGATTGCT3') and E6AS (5'AGTTGCAAACCAGACCTCAGG3') for exon 6, E7S (5'CCTGTGTTATCTCCTAGGTTG3') and E7AS (5'GCACAGCAGGCCAGTGTGCA3') for exon 7, and E8S (5'GACCTGATTTCCTTACTGCC3') and E8AS (5'TCTCCTCCACCGCTTCTTGT3') for exon 8. An initial cycle was performed for 5 min at 94 ℃; followed by 35 cycles of 40 s at 94 ℃, 40 s at 54 ℃, and 1 min at 72 ℃. The PCR products were sequenced using an Applied Biosystems 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, United States), and the same primers were used for both the PCR and for the DNA sequencing. All p53 mutations were confirmed by direct sequencing of both DNA strands.

Nested polymerase chain reaction

Genomic DNA was prepared from a tissue section and isolated by conventional phenol-chloroform extraction, ethanol precipitation, and was finally dissolved in 20 μL of sterile distilled water. HPV viral DNA was first amplified with consensus primers MY09 and MY11[19] followed by a second round of amplification with type-specific primers flanking the L1 region to identify the subtype. Ten microliters of the final PCR product were loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet-visible illumination. Appropriate negative and positive controls were included in each PCR reaction. A part of the β-actin gene in all samples was amplified to exclude false-negative results while DNA preparations from the SiHa cell (containing HPV16) were used as positive controls.

ISH

ISH for the detection of HPV type 16 DNA was performed using digenoxenin-labeled (DIG-labeled) oligonucleotide probes and a commercially available hybridization kit (Boehringer Mannheim, Indianapolis, IN). Briefly, the hybridizing probes were prepared by PCR amplification using HPV 16 type-specific primers with DIG-deoxyuridine triphosphate as a substrate according to the manufacturer's instructions[19]. The deparaffinized and rehydrated 5 μm sections were digested with proteinase K, rinsed with PBS, and dehydrated. The hybridization was performed in a humidified chamber at 48 ℃ for 16 h followed by a wash with sodium chloride-sodium citrate. Thereafter, the detection reagent (anti-DIG antibody conjugated with peroxidase) was applied to the sections and then the sections were incubated in diaminobenzidine solution to allow the signals to develop. After the signal development, the sections were counterstained with hematoxylin, rinsed briefly in absolute ethanol, mounted, and observed for signals under a microscope.

Preparation of RNA and real-time quantitative RT-PCR

Total RNA was extracted from the colorectal tumors after homogenization in 1 mL TRIzol reagent, followed by chloroform re-extraction and isopropanol precipitation. Three micrograms of total RNA from the colorectal tumor tissues were reverse transcribed using SuperScript Ⅱ Reverse Transcriptase (Invitrogen, CA, United States) and oligo d(T)15 primer. Real-time RT-PCR was performed in a final volume of 25 μL containing 1 μL of each cDNA template, 10 pmoles of each primer, and 12.5 μL of a SYBR-Green master mix. The primers were designed using ABI Prism 7000 SDS Software. Quantification was carried out using the comparative threshold cycle (CT) method and water was used as the negative control. An arbitrary threshold was chosen on the basis of the variability of the baseline. CT values were calculated by determining the point at which the fluorescence exceeded the threshold limit. CT was reported as the cycle number at this point. The average of the target gene was normalized to *18S rRNA* as the endogenous housekeeping gene.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software program (Version 11.0 SPSS Inc., Chicago, IL, United States). The χ^2 test, Fisher's exact test (two tailed), and Mann-Whitney test were applied for statistical analysis.

RESULTS

HPV16 DNA does indeed exist in a subset of colorectal tumors

Sixty-nine colorectal tumors were collected to evaluate HPV16 DNA. As shown in Figure 1, the presence of HPV16 DNA in colorectal tumors was determined by nested PCR using MY09/MY11 and specific primers, and 11 of 69 tumors (16%) possessed positive HPV16 DNA signals. The relationships between HPV16 DNA and clinicopathological features were statistically analyzed (Table 1). Our data showed that HPV16 DNA was present more frequently in stage I tumors than in late-stage tumors (66.7% for stage I *vs* 12.5% for stage Ⅱ, 9.5% for stage Ⅲ,

Table 1 Relationships between human papilloma virus 16 infection and clinical parameters of colorectal cancer patients *n* (%)

| Parameters	HPV16		
	Negative	**Positive**	***P* value**
Gender			0.782
Male (*n* = 35)	29 (82.9)	6 (17.1)	
Female (*n* = 34)	29 (85.3)	5 (14.7)	
Age (yr)			0.322
< 68 (*n* = 31)	28 (90.3)	3 (9.7)	
> 68 (*n* = 38)	30 (78.9)	8 (21.1)	
Smoking status			0.718
No (*n* = 49)	42 (85.7)	7 (14.3)	
Yes (*n* = 20)	16 (80.0)	4 (20.0)	
Stage			0.017
I (*n* = 6)	2 (33.3)	4 (66.7)	
II (*n* = 24)	21 (87.5)	3 (12.5)	
III (*n* = 21)	19 (90.5)	2 (9.5)	
IV (*n* = 18)	16 (88.9)	2 (11.1)	
Tumor site			0.554
Ascending colon (*n* = 22)	19 (86.4)	3 (13.6)	
Transverse colon (*n* = 5)	5 (100.0)	0 (0)	
Descending colon (*n* = 5)	3 (60.0)	2 (40.0)	
Sigmoid colon (*n* = 10)	9 (90.0)	1 (10.0)	
Rectum (*n* = 27)	22 (81.5)	5 (18.5)	

HPV: Human papilloma virus.
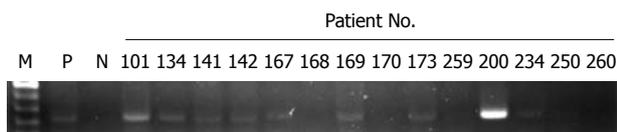
Figure 1 Representatives of positive and negative human papilloma virus 16 DNA detected by nested polymerase chain reaction in tumors of colorectal cancer patients. M: 100-bp ladder DNA marker; P: Positive control; DNA of SiHa cell line served as positive control for human papilloma virus 16, respectively; N: Negative control, the DNA template was replaced with distilled water.

Table 2 p53 mutation, p53 protein, E6 oncoprotein and clinical information in human papilloma virus 16 DNA-positive colorectal patients

Patient No.	Gender	Age (yr)	Stage	Site	p53		HPV16	
					Mutation	**IHC**	**ISH**	**E6**
101	F	65	I	d-colon	No	-	+	+
134	F	54	II A	Rectum	No	+	+	+
141	M	72	I	Rectum	No	-	+	+
142	F	56	II A	d-colon	Yes	++	+	+
167	M	70	III B	d-colon	Yes	+++	+	+
169	M	78	IV	Rectum	No	-	+	+
173	F	83	II A	s-colon	Yes	+	+	+
200	M	74	IV	a-colon	Yes	+	+	+
226	F	78	I	Rectum	No	-	-	-
234	F	76	I	a-colon	No	+	-	-
259	F	79	III B	Rectum	Yes	++	-	-

HPV: Human papilloma virus; IHC: Immunohistochemistry; ISH: *In situ* hybridization. -: No positive staining; +: 1%-10%; ++: 1%-50%; +++: > 50% positive cells.

Table 3 p21 and Mdm2 mRNA expression levels in tumor tissues and adjacent normal tissues of E6-positive colorectal patients

Variable	E6-positive (*n* = 8)			
	p53 wild-type (*n* = 4)	***P***	**p53-mutation (*n* = 4)**	***P***
P21 mRNA		0.025		0.061
Adjacent normal tissues	348.46 ± 143.89		241.12 ± 84.13	
Tumor tissues	49.62 ± 16.58		122.96 ± 45.28	
Mdm2 mRNA		0.032		0.043
Adjacent normal tissues	563.74 ± 252.28		541.50 ± 100.56	
Tumor tissues	111.60 ± 75.99		317.74 ± 137.59	

and 11.1% for stagev IV, $P = 0.017$). However, the presence of HPV16 DNA in colorectal tumors was not associated with other clinicopathological features including gender, age, smoking status, and tumor site (Table 1). To further confirm the presence of HPV16 DNA, ISH was conducted to determine the presence of HPV16 DNA in 11 colorectal tumor paraffin sections. ISH data indicated that 8 of the 11 tumors (72.7%) had positive HPV16 DNA signals (Table 2 and Figure 2). Collectively, these results clearly indicate that HPV16 DNA exists in a subset of colorectal tumors, at least in this study population.

E6 oncoprotein is expressed in HPV16 DNA-positive colorectal tumors and related to p53 inactivation

To explore whether HPV16 infection could be linked with colorectal cancer development, HPV16 E6 oncoprotein was evaluated by IHC in HPV16 DNA-positive colorectal tumors. Our data showed that E6 oncoprotein expression was detected in all HPV16 DNA-positive tumors (Table 2). We next examined whether E6 oncoprotein expressed in four p53 wild-type colorectal tumors (P101, P134, P141 and P169) could degrade p53 protein to produce tumors

with p53 negative immunostaining (Figure 2). IHC analysis clearly showed p53 negative immunostaining in three out of four colorectal tumors (P101, P141, and P169), but one tumor (P134) had p53 positive immunostaining (< 10%). The other four E6-positive tumors with p53 mutations (P142, P167, P173 and P200) still had p53 positive immunostaining (Figure 2). To explore whether p53 inactivation could have occurred in p53 wild-type E6-positive tumors, expression levels of the p53-downstream targets Mdm2 and p21 mRNA were decreased compared with adjacent normal tissues. Real-time RT-PCR analysis showed that Mdm2 and p21 mRNA expression levels in these E6-positive tumors with p53 wild-type were significantly lower than in their adjacent normal tissues ($P = 0.025$ for p21 mRNA; $P = 0.032$ for Mdm2 mRNA; Table 3). As expected, both gene mRNA expression levels in E6-positive tumors with p53 mutations were lower than in the adjacent normal tissues ($P = 0.043$ for Mdm2 mRNA); however, p21 mRNA levels were marginally different between tumors and adjacent normal tissues ($P = 0.061$). These results clearly indicate that E6 oncoprotein is expressed in HPV16 DNA-positive colorectal tumors and may be linked with p53 inactivation in these HPV16 DNA positive tumors, which had the *p53* wild-type gene.

P167   P169
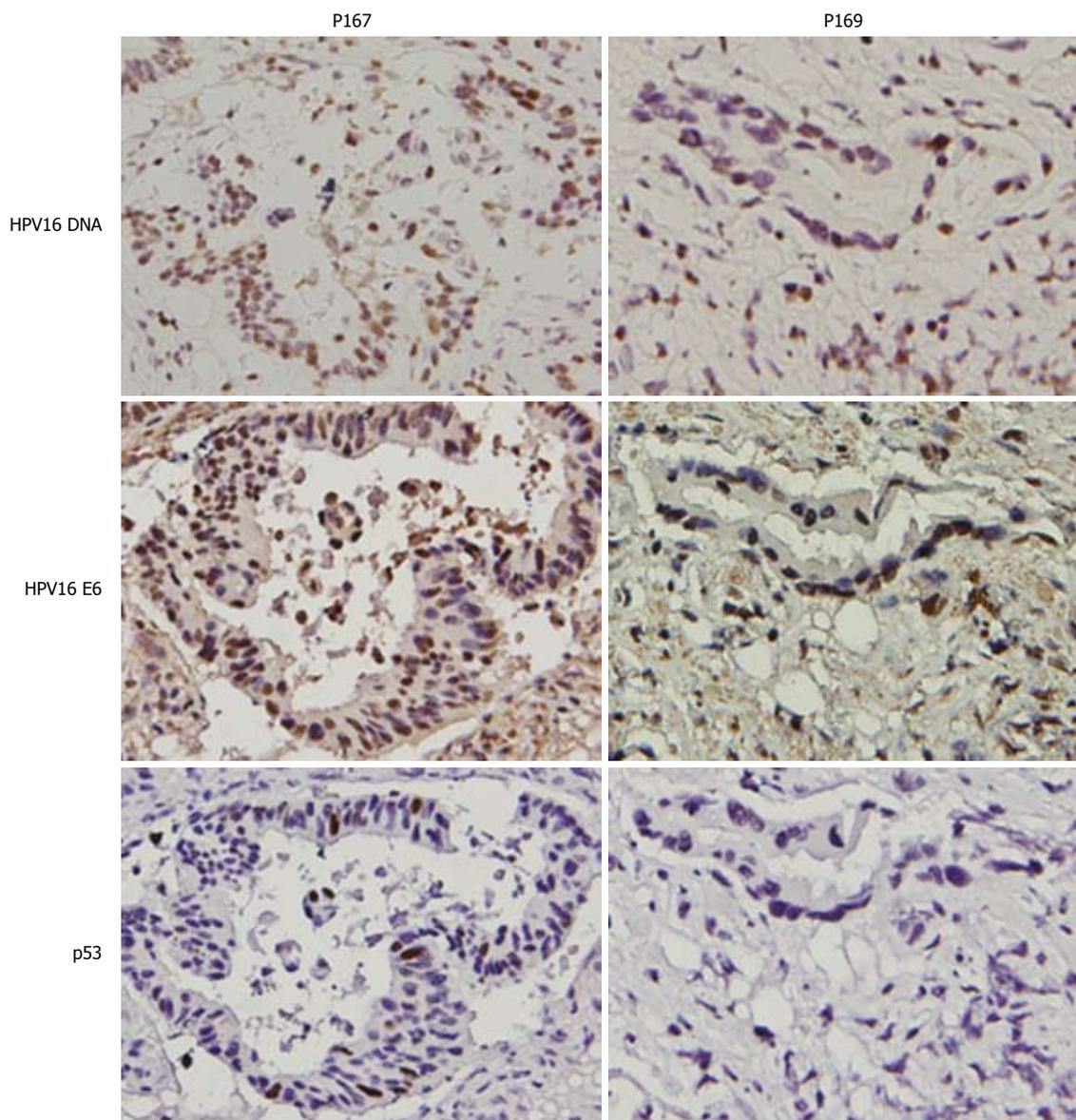
Figure 2 The representative reciprocal relationships between human papilloma virus 16 E6 and p53 immunostainings in human papilloma virus 16-infected colorectal (400 ×). HPV: Human papilloma virus.

E6 oncoprotein is expressed in endothelial cells and lymphocytes of colorectal tumors

To understand whether HPV-infected colorectal tumors could be mediated through blood circulation, E6 oncoprotein expression in normal parts of the colorectal tumors were examined by IHC (Figure 3). Our data showed that E6 oncoprotein is indeed expressed in endothelial cells of blood vessels and in lymphocytes infiltrating colorectal tumors. In addition, E6 oncoprotein was expressed in normal glands and dysplastic glands in colorectal tumors (Figure 3). These results seem to support the possibility that HPV16 infection in colorectal tumors may occur partially through blood circulation.

DISCUSSION

The presence of HPV is commonly detected by nested PCR in human tumors including colorectal tumors. In previous studies, only one report has shown the concomitant detection of HPV16 DNA in three colorectal tumors by nested-PCR and *in situ* PCR[15]. In the present study, nested PCR detected 8 of 11 HPV16-positive colorectal tumors and ISH confirmed this finding as well. Detection of HPV DNA by ISH had markedly lower sensitivity than *in situ* PCR, suggesting that the HPV16 DNA copy number in the tumors studied herein could be higher than previously reported[19]. In the current study, the association between HPV infection and tumor stage was observed; however, only 6 stage I patients were enrolled in this study. Since the sample size of stage I patients is so small, it might be biased to conclude that HPV infection might play a role in the development of colorectal cancer. In addition, among 4 of the 6 stage I tumors with HPV16 DNA, HPV infection was confirmed by ISH in only 2 tumors. Therefore, the association between HPV infection and tumor stage should be verified by a larger
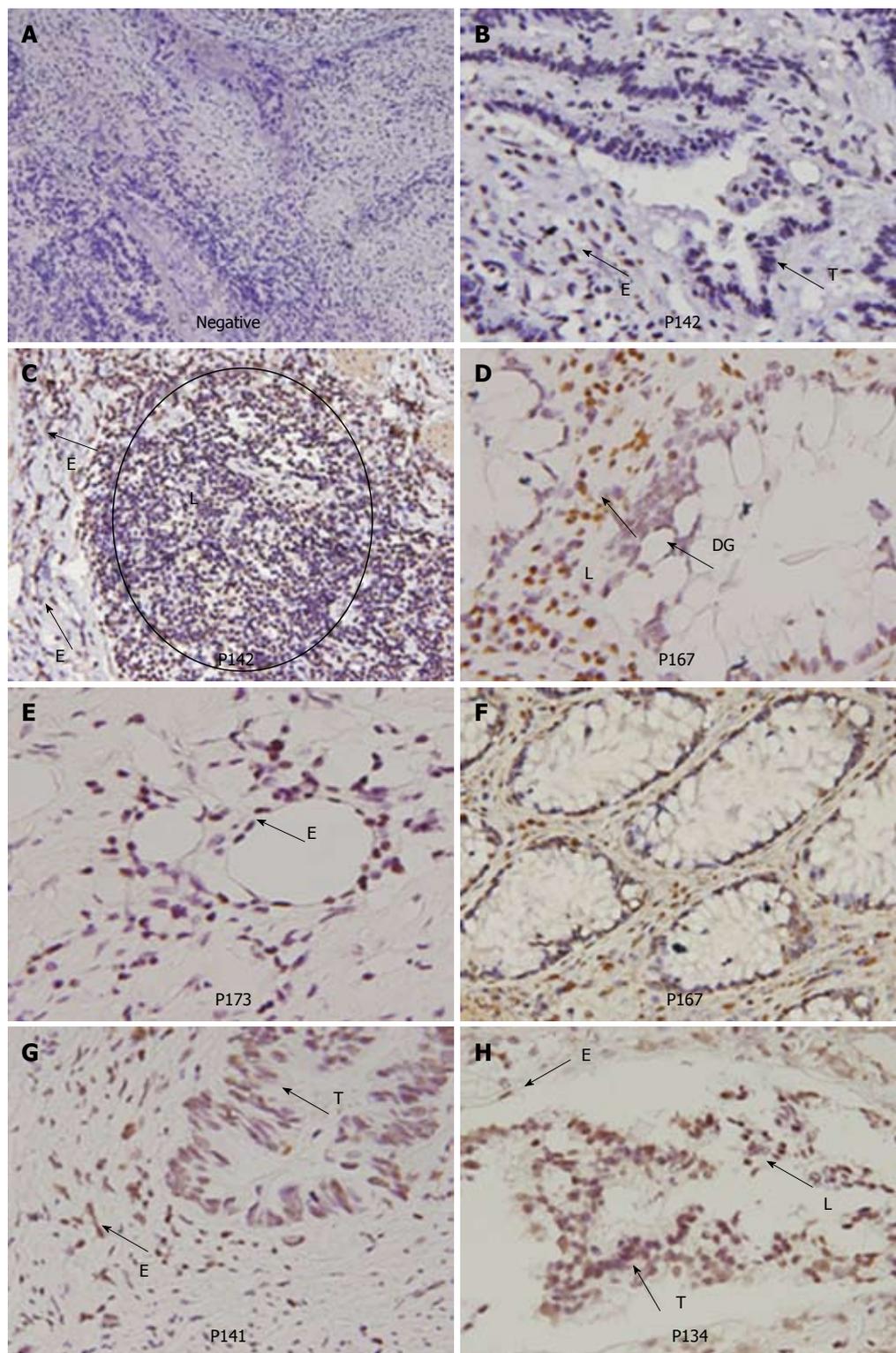
Figure 3 Immunohistochemical analysis of human papilloma virus 16 E6 protein in colorectal tumors and adjacent normal tissues. A: A negative result of immunostaining in tumor cells (100 ×); B: Human papilloma virus 16 (HPV16) E6 protein expressed in endothelial cells (400 ×); C: HPV16 E6 protein expressed in endothelial cells and lymphocytes (100 ×); D: HPV16 E6 protein expressed in lymphocytes and dysplastic gland (400 ×); E: HPV16 E6 protein expressed in endothelial cells and tumor cells (400 ×); F: HPV16 E6 protein expressed in normal gland (400 ×); G: HPV16 E6 protein in endothelial cells and Fibroblast (400 ×); H: HPV16 E6 protein in endothelial cells, lymphocytes and tumor cells (400 ×). E: Endothelial cells; T: Tumor cells; L: Lymphocytes; DG: Dysplastic gland.

study population.

To explore whether the existence of HPV16 DNA could be involved in colorectal cancer development, the presence of E6 and p53 proteins in tumor tissues and p21 and Mdm2 mRNA expression in tumors and adjacent normal tissues were evaluated in a subset of colorectal tumors (11 of 69, 16%). E6 oncoprotein expression was detected in 8 of the 11 HPV16 DNA-positive tumors.

Among these eight E6-positive tumors, p21 and Mdm2 mRNA expression levels were markedly decreased in four E6-positive tumors carrying the wild-type *p53* gene; however, no significant decrease in both gene mRNA expression levels was noted in the other four E6-positive tumors carrying the mutated *p53* gene. Therefore, these results clearly show that E6 oncoprotein may be involved in this small subset of colorectal cancer development *via* the inactivated p53 pathway (Table 3).

Sexual activity has been considered to be a major route of transmission for HPV resulting in genital cancers, such as cervical, anal, and oropharyngeal cancers[1,2]. However, there is evidence of HPV infections in infants and female university students who are virgins, revealing that HPV transmission *via* other routes than sexual intercourse may exist[3,13,14,20-25]. In addition, peripheral blood lymphocytes (PBLs) from healthy donors have been shown to be infected with HPV[13]. Therefore, it has been suggested that HPV infection in internal organ tissues might occur through blood circulation. Our previous lung cancer studies have indicated that HPV16/18 DNA and E6 oncoprotein not only exist in lung tumors but are also expressed in adjacent normal tissues including lymphocytes, endothelial cells, macrophages, and bronchial epithelial cells[26]. We therefore speculated that PBLs may first be infected by HPV to act as a mediator of HPV infection to lung tissues *via* blood circulation[14,26]. In the present study, IHC analysis clearly indicates that E6 oncoprotein was expressed in tumor-infiltrating lymphocytes of HPV16 DNA-positive colorectal tumors. In addition, E6 oncoprotein expression was also detected in the endothelial cells of the HPV16 DNA-positive colorectal tumors. These results seem to support our previous hypothesis that HPV infection in lung tumor tissues may be mediated through blood circulation and not as a direct contact transmission in cervical and oropharyngeal tumor tissues[14,19,26].

To our knowledge, this is the first report to indicate that HPV16 E6 oncoprotein may downregulate p21 and Mdm2 transcription *via* inactivation of p53 in the involvement of colorectal cancer development. Similar observations of E6 oncoprotein expression in adjacent normal lung and colorectal tissue cells seem to support the possibility that HPV infection in colorectal tumors might be mediated through blood circulation. Notably, even though our present study provides support for the association between HPV infection and colorectal cancer, the involvement of HPV infection in colorectal cancer development is limited to a small subset of the population.

COMMENTS

Background
Human papilloma virus (HPV) DNA integration into the host chromosome plays a crucial role in HPV-associated tumorigenesis. When HPV DNA was spliced at E2, E6 and E7 oncoproteins were expressed to inactivate p53 and Rb pathways. A high risk of HPV16/18 infection has been documented to be involved in the development of cervical and anal genital cancers. Among non-genital cancers, the association of oropharyngeal cancer with HPV16/18 infection was recently evidenced. However, other non-genital cancers, including lung, breast, and colorectal cancers have not yet been identified.

Research frontiers
The authors provide the evidence to indicate that HPV16 may be involved in a small subset of colorectal cancer development. Therefore, HPV vaccination not only prevents cervical cancer but also reduces HPV-associated colorectal cancer development.

Innovations and breakthroughs
The association of HPV infection with colorectal cancer has been extensively investigated; however, no strong evidence supports the involvement of HPV in colorectal cancer development. Herein, nested-polymerase chain reaction and *in situ* hybridization were used to detect the presence of HPV16 DNA in colorectal tumors. Immunohistochemical data further showed that E6 oncoprotein is expressed in HPV16 DNA-positive tumors and E6 expression was negatively correlated with p53 expression. These results suggest that HPV16 might contribute to a small subset of colorectal cancer development.

Applications
HPV infection is not only involved in cervical and oropharyngeal cancers but is also linked with internal organ cancers, such as lung and colorectal cancers. Therefore, blood transmission of HPV infection in internal organs might be noted in public.

Peer review
The authors present an interesting work on the expression of HPV16 E6 oncoprotein in colorectal cancer. The manuscript is generally well written. The topic is very interesting and to some extent provocative. The manuscript is well structured and the cited literature is comprehensive and up-to-date.

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BRIEF ARTICLE

Excisional hemorrhoidal surgery and its effect on anal continence

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Abstract

AIM: To investigate the role of anal cushions in hemorrhoidectomy and its effect on anal continence of the patients.

METHODS: Seventy-six consecutive patients (33 men and 43 women) with a mean age of 44 years were included. They underwent Milligan-Morgan hemorrhoidectomy because of symptomatic third- and fourth-degree hemorrhoids and failure in conservative treatment for years. Wexner score was recorded and liquid continence test was performed for each patient before and two months after operation using the techniques described in our previous work. The speed-constant rectal lavage apparatus was prepared in our laboratory. The device could output a pulsed and speed-constant saline stream with a high pressure, which is capable of overcoming any rectal resistance change. The patients were divided into three groups, group A (< 900 mL), group B (900-1200 mL) and group C (> 1200 mL) according to the results of the preoperative liquid continence test.

RESULTS: All the patients completed the study. The average number of hemorrhoidal masses excised was 2.4. Most patients presented with hemorrhoidal symptoms for more than one year, including a mean duration of incontinence of 5.2 years. The most common symptoms before surgery were anal bleeding ($n = 55$), prolapsed lesion ($n = 34$), anal pain ($n = 12$) and constipation ($n = 17$). There were grade III hemorrhoids in 39 (51.3%) patients, and grade IV in 37 (48.7%) patients according to Goligher classification. Five patients had experienced hemorrhoid surgery at least once. Compared with postoperative results, the retained volume in the preoperative liquid continence test was higher in 40 patients, lower in 27 patients, and similar in the other 9 patients. The overall preoperative retained volume in the liquid continence test was 1130.61 ± 78.35 mL, and postoperative volume was slightly decreased (991.27 ± 42.77 mL), but there was no significant difference ($P = 0.057$). Difference was significant in the test value before and after hemorrhoidectomy in group A (858.24 ± 32.01 mL *vs* 574.18 ± 60.28 mL, $P = 0.011$), but no obvious difference was noted in group B or group C. There was no significant difference in Wexner score before and after operation (1.68 ± 0.13 *vs* 2.10 ± 0.17, $P = 0.064$). By further stratified analysis, there was significant difference before and 2 months after operation in group A (2.71 ± 0.30 *vs* 3.58 ± 0.40, $P = 0.003$). In contrast, there were no significant differences in group B or group C (1.89 ± 0.15 *vs* 2.11 ± 0.19, $P = 0.179$; 0.98 ± 0.11 *vs* 1.34 ± 0.19, $P = 0.123$).

CONCLUSION: There is no difference in the continence status of patients before and after Milligan-Morgan hemorrhoidectomy. However, patients with preoperative compromised continence may have further deterioration of their continence, hence Milligan-Morgan hemorrhoidectomy should be avoided in such patients.

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Key words: Anal cushion; Anal incontinence; Liquids continence test; Wexner score; Hemorrhoidectomy

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INTRODUCTION

Hemorrhoids are very common and it occurs in men and women of all ages. It is estimated that 50% of the people older than 50 years have hemorrhoids symptoms at least for a period of time[1]. The most common symptoms include rectal bleeding, pain, anal irritation and anal mass prolapse and a disrupted quality of life. There has been much speculation over the years as to the nature of "hemorrhoids". It is now generally accepted that "hemorrhoids" are a disorder of the anal cushions[2]. Thomson demonstrated that in patients suffering from hemorrhoids, the specialized "cushions" of submucosal tissue lining the anal canal slide downwards, together with the anal mucosa, due to the fragmentation of Parks' ligament[3-5]. This means that hemorrhoids result from distal enlargement of the anal cushions. The anal cushions are connective tissue complexes that contain smooth cells and vascular channels; they are thought to provide an effective tight seal to close the anal in concert with the internal anal sphincter[6].

For patients with grades III and IV hemorrhoids, surgical excision remains the most common choice of treatment. Two main approaches have been used, one removes the cushions (e.g., Milligan-Morgan hemorrhoidectomy) and the other retains the cushion (e.g., stapled hemorrhoidopexy or procedure for prolapse and hemorroids). The expensive stapled hemorrhoidopexy has become a widely accepted alternative to excisional hemorrhoidectomy for treating the third- and fourth-degree hemorrhoids in China over the recent decade, it even has a trend to replace the traditional hemorrhoidectomy[7]. According to Thomson's theory, impairment of the anal cushions may lead to anal incontinence. Some previous studies[8-13] indicate that hemorrhoidectomy might be complicated with fecal incontinence. Therefore, many non-excisional options have become available to overcome the disadvantages of traditional surgery, which have given rise to dispute around the world. However, to our knowledge, there has been no direct evidence supporting the function of the anal cushion. Our study aims to define the role of the anal cushions in hemorrhoidectomized patients by performing a liquid continence test simulating analorectal continence of liquid stool and using the Wexner score (the Cleveland clinic continence scoring system)[14,15].

MATERIALS AND METHODS

Patients

Consecutive patients with hemorrhoids were included in the study. Inclusion criteria for the cohort were: the existence of symptomatic third- and fourth-degree hemorrhoids, and failure in conservative treatment for years and intention for Milligan-Morgan hemorrhoidectomy. Patients younger than 18 or older than 80 years, who had experienced concomitant anal diseases (fissure, abscess, fistula, inflammatory bowel disease, rectal cancer) were excluded. Eligible patients were asked for signed informed consent. The study was approved by the local ethics committee.

Eighty patients who referred to our hospital between April 2005 and September 2010 were recruited. Four patients, who lost to follow-up and did not complete the second Wexner scoring and liquid continence test, were excluded. Eventually, 76 patients were eligible and completed the study. The demographic and clinical data, and the results of liquid continence test were obtained. The anal continence was assessed using the Wexner scoring system.

Liquid continence test was performed and Wexner score was recorded for each patient before and two months after operation. The patients were divided into three groups: group A (< 900 mL), group B (900-1200 mL) and group C (> 1200 mL) according to the retained volume in the liquid continence test done before operation.

Each patient underwent the standard Milligan-Morgan hemorrhoidectomy using conventional instruments for dissection and a monopolar coagulator for hemostasis by experienced surgeons.

Liquid continence test

Liquid continence test was performed preoperatively in all the patients. At 2 mo follow-up after operation, wounds were healed in all the patients. The same tests were repeated two months after surgery (60 ± 3 d).

This test was performed using the techniques described in our previous work[16]. The speed-constant rectal lavage apparatus was prepared in our laboratory[16]. The device could output a pulsed and speed-constant saline stream with a high pressure, capable of overcoming any rectal resistance change.

The first liquid continence test was performed at the preoperative days 1 and 2. Patients were advised to empty their rectums before the examination. The patient sat on the device. After a F16 balloon urethral catheter was introduced into the rectum about 8-cm deep, the balloon was inflated with 5 mL air. The warm saline (37 ℃) was infused at a rate of 60 mL/min. The patients were instructed to hold the liquid as long as possible. If the device was alarmed when a leakage amount reached 10 mL or the infusing liquid reached the maximum (1500 mL), the test stopped. The total volume retained was recorded. After the end-point was recorded, the balloon was deflated and the catheter was extracted.

Variables	Values
Age (yr)[1]	44 (22-72)
Gender (male/female)	33/43
Chief complaints, *n* (%)	
Prolapse	32 (42.1)
Anal bleeding	55 (72.3)
Anal pain	12 (15.8)
Constipation	17 (25.4)
Hemorrhoids stage, *n* (%)	
Third-degree	39 (51.3)
Fourth-degree	37 (48.7)
Resected piles (*n*)[2]	2.4 ± 0.3
Disease duration (yr)[2]	3.6 ± 1.1

[1]Data are median (range); [2]Data are means ± SD.

Wexner score

Anal incontinence was assessed using the Wexner grading system[14]. Wexner score contains three items about the type and frequency of incontinence (scored from zero to four) and items on pad usage and lifestyle alteration (both scored from zero to four). Data were collected by physicians through a patient interview.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software. The data were expressed as mean ± SD or median (range). Continuous data were compared using paired Student's *t* test. Difference was considered to be significant when the *P* value was < 0.05.

RESULTS

The demographics and clinical details of the 76 patients are shown in Table 1. The mean age of patients was 44 years (range: 22-72 years) and the male to female ratio was 1:1.3. The number of hemorrhoidal masses excised varied from 2-4 (mean: 2.4 ± 0.3). The mean duration of incontinence was 5.2 years. Most patients had hemorrhoidal symptoms for more than one year, and some patients even for more than 20 years. The most common symptoms observed in the patients before surgery was anal bleeding (55 cases), prolapsed lesion (34 cases), anal pain (12 cases) and constipation (17 cases) (Table 1). Thirty-nine (51.3%) patients had grade Ⅲ hemorrhoids, and 37 patients (48.7%) had grade Ⅳ hemorrhoids according to the Goligher classification. Five patients experienced hemorrhoid surgery at least once.

Compared with the postoperative results, the retained volume in the preoperative liquid continence test was higher in 40 patients, lower in 27 patients, and similar in the rest 9 patients. The overall preoperative volume in the liquid continence test was 1130.61 ± 78.35 mL, and postoperative values were slightly decreased (991.27 ± 42.77 mL), but there was no significant difference (*P* = 0.057).

According to the results of preoperative test, patients were divided into three groups: 17 patients in group A (<

900 mL), 26 in group B (900-1200 mL) and 32 in group C (> 1200 mL) (Table 2). Interestingly, significant difference was found in the test results before and after hemorrhoidectomy in group A (858.24 ± 32.01 mL *vs* 574.18 ± 60.28 mL, *P* = 0.011), but no obvious difference was noted in group B or group C (Table 2).

There was no significant difference in the Wexner score before and after operation (1.68 ± 0.13 *vs* 2.10 ± 0.17, *P* = 0.064; Table 2).

By further stratified analysis, there was significant difference in the Wexner score before and two months after operation in group A (2.71 ± 0.30 *vs* 3.58 ± 0.40, *P* = 0.003). In contrast, there were no significant differences in group B or group C (1.89 ± 0.15 *vs* 2.11 ± 0.19, *P* = 0.179; 0.98 ± 0.11 *vs* 1.34 ± 0.19, *P* = 0.123; Table 2).

DISCUSSION

To evaluate accurately the anal continence is still a clinical challenge. Parks[17] pointed out that it is difficult to evaluate postoperative anal continence due to the flaws related to subjective and objective factors. We, therefore, used liquid continence test and Wexner scoring system in combination to better assess the anal continence status. The liquid continence test could simulate liquid stool; compared with anorectal manometry, it is more applicable, which can yield objective assessment with quantitative data[16,18,19]. The Wexner Continence Grading Scale has been widely used for evaluating anal continence[20-22]. It is convenient in practice and easily acceptable by the patients. Consequently, our assessments based on liquid continence test and questionnaire scoring system, are likely to be more reliable.

In our study, the mean Wexner Continence Grading Scale did not vary significantly after surgery in the overall patients. By further subgroup analysis, after hemorrhoidectomy, the Wexner score significantly increased in patients with preoperative continence defect or subclinical incontinence (group A), while no significant difference was observed in the group with normal anal continence (groups B and C). Similar results were obtained by means of the liquid continence test. While the liquid continence test and Wexner scoring system yielded the similar results, the former is more direct and objective. Baxter *et al*[23] insisted that fecal incontinence is manifested as a symptom, so any evaluation of incontinence must be built on the perception of the patient. This is one of the reasons why we prefer the liquid continence test in the evaluation.

What is the role of anal cushion in hemorrhoidectomy? In spite of the high incidence of hemorrhoidal diseases worldwide, some aspects of its pathophysiology still remain unknown. According to Thomson's attractive theory, the anal cushions serve as a conformable plug to ensure complete closure of the anal canal and contribute to the anal continence mechanism[3]. Hemorrhoidectomy is associated with the removal of the anal cushions, and may occasionally lead to anal incontinence[7,11]. Jóhannsson *et al*[12] concluded from his questionnaire that 29% of

Table 2 Results of liquid continence test before and after hemorrhoidectomy and Wexner score assessments (mean)

	Liquid continence test (pre-operation) (mL)	Liquid continence test (post-operation) (mL)	P value	Wexner score (pre-operation)	Wexner score (post-operation)	P value
Total patients	1130.61 ± 78.35	991.27 ± 42.77	0.057	1.68 ± 0.13	2.10 ± 0.17	0.064
Group A (n = 17)	858.24 ± 32.01	574.18 ± 60.28	0.011	2.71 ± 0.30	3.58 ± 0.40	0.003
Group B (n = 26)	977.96 ± 15.96	927.31 ± 53.23	0.061	1.89 ± 0.15	2.11 ± 0.19	0.179
Group C (n = 33)	1391.21 ± 16.95	1257.79 ± 51.53	0.124	0.98 ± 0.11	1.34 ± 0.19	0.123

the patients reported the incontinence after hemorrhoidectomy. Thekkinkattil *et al*[24] demonstrated that the cushion: canal ratio was reduced in patients with idiopathic fecal incontinence. However, the theory is controversial by the fact that submucosal hemorrhoidectomy does not yield better functional outcome than excisional hemorrhoidectomy[25]. Our study revealed that hemorrhoidectomy (excised anal cushions) did not impair the function of anal continence. Our findings agree with a previous report[26] that no incontinence occurred after conventional hemorrhoidectomy. However, there is still much uncertainty regarding the role of anal cushion in fecal incontinence. It is important to note, although no obvious change was observed in anal continence in the patients after excising the anal cushion, further subgroup analysis showed that the patients with a lower value of liquid continence test (< 900 mL) after surgery did impair the fecal continence. This may be partly attributed to the fact that this group of patients had been complicated with continence defect or subclinical incontinence.

Our data support that the traditional hemorrhoidectomy, which necessitates excision of anal cushions, is a safe procedure for patients with normal fecal continence. From the perspectives of cost-effectiveness, the traditional hemorrhoidectomy should be recommended, especially in the developing countries. Nevertheless, surgeons should keep in mind that this kind of surgery may increase the risk of complicated anal incontinence in the patients with anal function defect or subclinical incontinence. Best of all, this study enhances the awareness of surgeons that preoperative evaluation of hemorrhoid patients is important regarding the choice of surgical procedure.

Our study had several limitations, such as a small sample size, short follow-up, and saline continence test could not assess the solid stool, which might result in improper findings. Further studies are being conducted to work out an objective test for solid and flatus stool, and compare the anal cushion preserving and non-preserving procedures as well.

In conclusion, removing anal cushions does not obviously impair the fecal continence in patients with a proper indication for the operation, and therefore is a safe procedure. It is not necessary to pay excessive attention to anal cushion in hemorrhoid patients. Thorough investigations should be carried out on anal continence so as to prevent the occurrence of postoperative complications.

COMMENTS

Background
Traditional hemorrhoidectomy is tended to be replaced by stapled hemorrhoidopexy with anal cushion retained, because the anal cushion theory considers that the removal of the anal cushions will damage the anal rectal control function. In this study, water retention test and Wexner scoring were used to observe the changes of the anus and rectum control function in the patients treated with traditional hemorrhoidectomy.

Research frontiers
The authors evaluated the anal continence in the patients with hemorrhoids before and after operation using liquid continence test and Wexner scoring system in combination so as to define the role of anal cushions in hemorrhoidectomy.

Innovations and breakthroughs
To evaluate accurately the anal continence is still a clinical challenge, and it is especially difficult to evaluate the postoperative anal continence due to flaws related to subjective and objective factors. The authors, therefore, used liquid continence test and Wexner scoring system in combination in an attempt to better assess the anal continence. The liquid continence test could simulate liquid stool, thus yielding objective assessment with quantitative data. The Wexner scoring system is convenient in practice and easily acceptable by the patients. Consequently, the assessments obtained by this study based on liquid continence test and questionnaire scoring system, are likely to be more reliable. The authors also found that removing anal cushions does not obviously impair the fecal continence in patients with a proper indication for the traditional hemorrhoidectomy, and therefore it is a safe procedure.

Applications
According to this study, that the traditional hemorrhoidectomy, which necessitates excision of anal cushions, is a safe procedure for patients with normal fecal continence. From the perspectives of cost-effectiveness, the traditional hemorrhoidectomy should be recommended, especially in the developing countries. Nevertheless, surgeons should keep in mind that this kind of surgery may increase the risk of complicated anal incontinence in the patients with anal function defect or subclinical incontinence

Peer review
This study provides new evidences on the safety of Milligan-Morgan hemorrhoidectomy which is less expensive for patients. Some elements may substantiate the study, such as fecal continence test for not only liquid and but also for solid and flatus stools, and comparison between anal cushion preserving and non-preserving procedures.

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CASE REPORT

Rapidly deforming gastric carcinosarcoma with osteoblastic component: An autopsy case report

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Abstract

Carcinosarcomas are rare, malignant, biphasic tumors simultaneously comprising carcinoma and sarcoma in a single tumor. We present an extremely rare case of gastric carcinosarcoma with an osteoblastic component that drastically changed its shape within 2 mo. A 59-year-old male patient presented to the emergency outpatient unit with a complaint of black stool. Gastrointestinal endoscopy showed an ulcerated mass in the cardia of the lesser curvature of the stomach. Biopsy specimens revealed only adenocarcinoma. Two months later, the ulcerated lesion drastically changed its shape into an exophytic tumor. Total gastrectomy was performed. In the resected specimen, the gastric tumor contained both adenocarcinoma and sarcoma components with lace-like osteoid. The patient died 7 mo after the operation, and an autopsy was performed. In the autopsy, widespread metastases were present in the liver, lung, lymph nodes and peritoneum. In this report, we describe a case of gastric carcinosarcoma and presume its tumorigenesis based on the autopsy findings.

Key words: Carcinosarcoma; Stomach; Osteoblastic differentiation; Autopsy; Tumorigenesis

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INTRODUCTION

Carcinosarcomas are rare, malignant, biphasic tumors simultaneously comprising carcinoma and sarcoma in a single tumor. Although carcinosarcomas of the esophagus are most frequently observed in the upper gastrointestinal tract[1], they are very rarely found in the stomach. We performed a keyword search of the literature for gastric carcinosarcoma. Published articles, limited to English abstracts indexed primarily in the PubMed database, through to 2012, were reviewed. Until 2012, at least 46 cases of gastric carcinosarcoma have been reported[2-20]; most of them in Japanese patients. **In this article, we** present a rare case of gastric carcinosarcoma with an osteoblastic component that drastically changed its shape.

CASE REPORT

Case history

A 59-year-old Japanese male patient presented to the emergency outpatient unit with a complaint of black stool. He was an office worker. He had no apparent past
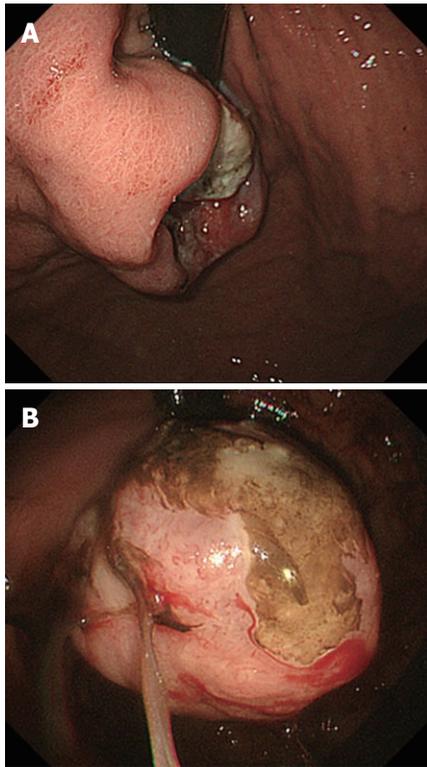
Figure 1 Retroflexed endoscopic view of the lesion in the gastric cardia. A: Ulcerated lesion; B: Two months later, the lesion changed its shape into an exophytic mass.


Figure 2 Macroscopic view of the resected specimen. A: An exophytic tumor located in the gastric cardia; B: Cross section of the tumor.

medical history and took no medications. There was no family history of malignant neoplasm. On physical examination, the abdomen was flat and not tender. Laboratory data were normal with the exception of a white blood cell count of 13 100/μL and an elevated serum carcinoembryonic antigen (CEA) level of 12.5 ng/mL (normal, < 5.0 ng/mL).

Gastrointestinal endoscopy showed an ulcerated mass in the cardia of the lesser curvature of the stomach (Figure 1A). The esophagogastric junction was not involved with the tumor. Biopsy from the lesion showed tubular adenocarcinoma. Two months later, upper gastrointestinal endoscopy was performed again and showed that the ulcerated lesion had drastically changed its shape into an exophytic mass (Figure 1B). Although the patient had multiple liver metastases, palliative total gastrectomy was performed to control gastric bleeding and ameliorate anemia.

After the palliative operation, systemic chemotherapy with S-1 and cisplatin was performed. As a result of tumor progression, the patient received second-line chemotherapy of irinotecan (CPT-11) and mitomycin C. Although three cycles of second-line chemotherapy were performed, liver metastases continued to develop. His general condition gradually worsened, and he died 7 mo after the operation. An autopsy was performed.

Pathological findings

On macroscopic examination of a 27 cm × 21 cm total gastrectomy specimen, an 11.5 cm × 10.5 cm × 5.0 cm

exophytic tumor was found in the cardia (Figure 2A). Erosion and necrosis were observed on the tumor surface. The cut surfaces of the tumor were tan-gray and fleshy (Figure 2B).

On light microscopy, the tumor contained both epithelial and spindle cell components. Spindle cell clusters and fascicles with the appearance of stromal sarcomas were observed in large regions. A small amount of lace-like osteoid was found in the sarcoma component (Figure 3A). Irregular fused glands with cuboidal and columnar cells appearing to overlap these regions were observed (Figure 3B). The spindle cells generally had coarse chromatin, elongated oval nuclei, small nucleoli, pale eosinophilic cytoplasm, and frequent mitotic activity. In adjacent regions of the tumor, dysplastic glands and goblet cell metaplasia were seen within the mucosa. No *Helicobacter pylori* (*H. pylori*) was observed. The tumor infiltrated the subserosal layer. Lymph node metastases with an adenocarcinoma component were detected in seven of 22 resected lymph nodes. Venous and lymphatic involvements with adenocarcinoma components were also observed. Immunohistochemically, cytokeratin AE1/AE3 positivity was observed in the tubular adenocarcinoma component (Figure 4A). There was no staining with cytokeratin AE1/AE3 in spindle cells. In spindle cells, positive staining with vimentin and negative staining with desmin, smooth muscle actin, c-kit, S-100, CD34, and HHF-35 was observed (Figure 4B). Widespread positive nuclear staining with p53 and more limitedly with Ki-67 was observed in both glandular and spindle tumor cells.

In the autopsy, widespread metastases were present in the liver, lungs, lymph nodes, and peritoneum. Although liver metastases showed both adenocarcinoma and spindle cell components, only the adenocarcinoma compo-
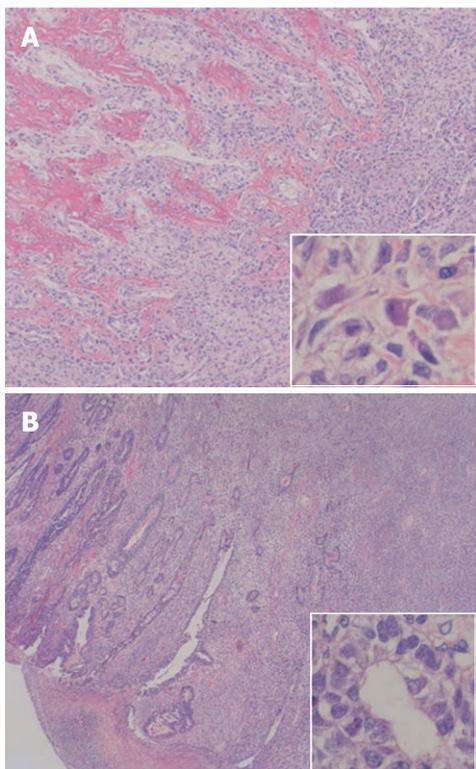
Figure 3 Representative microphotographs. A: Hematoxylin and eosin stain of the tumor (4 ×), a large part of the tumor comprised spindle cells producing lace-like osteoid; B: Tubular adenocarcinoma coexisting in the tumor. High-power view of tumor cells (40 ×) is shown in the insets.
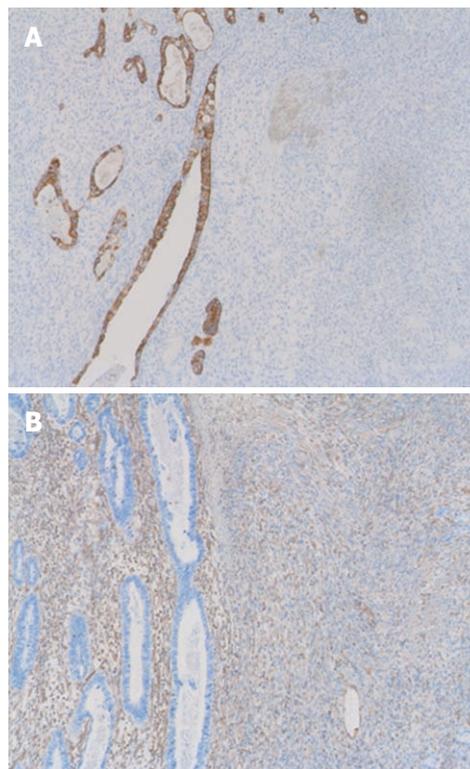

Figure 4 Immunohistochemical staining of the tumor. A: Cytokeratin AE1/AE3 staining showing positive expression in the adenocarcinoma component but not in the sarcomatous component (4 ×); B: Vimentin demonstrating opposite staining pattern (4 ×).

nent was present in lung metastases. Carcinosarcoma and pulmonary thromboembolism were the major causes of death in this case.

DISCUSSION

Gastric carcinosarcomas are rare tumors. They have also been referred to in the literature as sarcomatoid carcinomas. The first report of sarcomatoid carcinoma of the stomach was by Queckenstadt in 1904. Additional cases have been subsequently reported[2-19]. In 2007, Ikeda *et al*[20] reported one case and reviewed 43 cases from the literature. Until 2012, at least 46 cases of gastric carcinosarcoma have been reported; most of them in Japanese patients. We reported gastric carcinosarcoma containing an osteoblastic component, which is extremely rare. Based on our knowledge, there has been only one report of gastric carcinosarcoma with osteoblastic differentiation[14]. No previous report has described gastric carcinosarcoma as a tumor that rapidly deformed within 2 mo.

The histogenesis of biphasic tumors remains unclear. Some researchers have argued that a primary carcinoma stimulates excessive stromal proliferation, resulting in a carcinosarcoma[21]. Other researchers, however, are of the opinion that the spindle cell component reflects anaplasia within the carcinoma[4,22-24].

There are findings supporting the idea that gastric biphasic tumors are epithelial in origin[3,5,11]. In a number of studies, cells with an intermediate appearance between

that of epithelial and sarcomatous components have been seen, and these cells have been reported to stain positively with epithelial differentiation markers such as CEA. However, there have been cases in which spindle cells were differentiated as entirely smooth muscle or cartilage. In the literature, gastric carcinosarcomas differentiated as rhabdomyoblastic, neuroblastic, or osteoblastic have been reported[14,25-27]. A combination of carcinosarcoma and carcinoid tumor with neuroendocrine malignancy was reported in two cases[13,25]. Therefore, it may be that in their beginning, these tumors developed with diverse differentiation of a multipotent stem cell.

The histological findings observed in our case may support the theory that gastric biphasic tumors are epithelial in origin. All biopsy specimens obtained at the initial endoscopy showed only adenocarcinoma. However, in the surgically resected specimen, the spindle cell component occupied > 90% of the tumor, and a small number of adenocarcinoma cells were observed in the peripheral zone of the tumor. In the autopsy, the liver metastases that had been detected in the ante-mortem examination were composed of both spindle cell and adenocarcinoma components. In contrast, small metastases of the lung first diagnosed at autopsy showed only adenocarcinoma. Although these differences in tumor components at each metastatic site might have resulted from differences in affinities of each tumor component for lymphatics or veins, we believe that the development of the adenocarcinoma component might have preceded

that of the sarcoma component in our case. Rapid proliferation of the sarcoma component may have accounted for the drastic deformation of the tumor in our case. Although the tumorigenesis of this lesion is interesting from the perspective of epithelial-mesenchymal transition[28,29], a more sophisticated approach will be required to elucidate this problem.

From a clinical standpoint, gastric carcinosarcomas share clinicopathological features with gastric adenocarcinomas. The age range of the 46 cases previously reported was 29 to 83 years with a mean of 56 years[8,20,29]. There were 33 male and 13 female patients. Tumors that showed polypoid growth and ulcerated lesions were present in 22 and 19 cases, respectively. In our patient, the tumor exhibiting the ulcerative lesion rapidly changed its shape into an exophytic mass and was located in the cardia.

Gastric carcinosarcomas have a low incidence of metastasis, but mortality is high[5,11,13,14,25,26,30]. Approximately 50% of previously reported patients with gastric carcinosarcoma died of the disease within 6 mo[8,20,27]. In those cases that metastasize, the metastases may show pure carcinoma, pure sarcoma, or a mixture of both[16]. In our case, both components were present.

Histologically, carcinosarcomas include epithelial and sarcomatous regions, and the two tumor components may be separate or overlapping[3,5,13,14,25,26]. The tumor is often accompanied by atrophy, dysplasia, and intestinal metaplasia[5,11]. Several types of differentiation of the sarcoma component have been reported, including smooth muscle, cartilage, striated muscle, and neuroendocrine cells. However, production of osteoid by the sarcoma component is extremely rare. We observed staining with cytokeratin AE1/AE3 only in the epithelial component in the case presented. Vimentin positivity and production of lace-like osteoid suggested that spindle cells had differentiated as osteosarcoma. In our patient, widespread p53 and Ki-67 positivity confirmed the high proliferation and malignant potential of this tumor. *H. pylori* may not be strongly associated with carcinosarcoma[31,32].

In summary, we reported a case of gastric carcinosarcoma with an osteoblastic component that rapidly deformed within 2 mo. Carcinosarcoma of the stomach is a rare tumor and has a poor prognosis. It is impossible to establish standard therapy for this rare disease with a large-scale clinical trial, therefore, detailed descriptions and collections of individual cases should be continued.

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CASE REPORT

Cerebral lipiodol embolism after transarterial chemoembolization for hepatic carcinoma: A case report

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Abstract

We report a case of cerebral lipiodol embolism (CLE) after transarterial chemoembolization (TACE) for unresectable hepatic carcinoma (HCC). A 54-year-old man with unresectable HCC underwent TACE *via* the right hepatic artery and right inferior phrenic artery using a mixture of 40 mg pirarubicin and 30 mL lipiodol. His level of consciousness deteriorated after TACE, and non-contrast computed tomography revealed a CLE. The cerebral conditions improved after supportive therapy. The complication might have been due to hepatic arterio-pulmonary vein shunt caused by direct invasion of the tumor. Even though CLE is an uncommon complication of TACE, we should be aware of these rare complications in patients with high risk factors.

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Key words: Hepatic carcinoma; Cerebral lipiodol embolism; Chemoembolization

INTRODUCTION

Hepatic carcinoma (HCC) is one of the leading causes of cancer-related death. It has a tendency to invade the tissue around the tumor, such as diaphragm, portal and hepatic veins, which may result in formation of hepatic arteriopulmonary vein or hepatic arteriovenous shunts. Transarterial chemoembolization (TACE) is one of the most common treatment modalities as a palliative and preoperative method for patients with advanced HCC. Although various complications of TACE have been reported, cerebral lipiodol embolism (CLE) after TACE is rare. In this paper, we report a case of CLE after TACE for advanced HCC.

CASE REPORT

A 54-year-old man was admitted with right upper quadrant pain. He was a hepatitis B virus carrier for 30 years. The α-fetoprotein level was 1200 ng/mL. Enhance computed tomography (CT) revealed a 13-cm mass of the right liver lobe. These clinical signs indicated that the patient had unresectable HCC and Child-Pugh class A. As revealed by angiography, the huge hypervascular tumor located in the right liver was supplied by the right hepatic artery (RHA) and right inferior phrenic artery (RIPA) without arteriovenous shunt. TACE was performed *via* the RHA and RIPA using a mixture of 40 mg pirarubicin and 30 mL lipiodol. Toward the end of the procedure, the lipiodol was deposited in the tumor densely. The embolism process was monitored by fluoroscopy all the way and no abnormal flow of the lipiodol was found. Twenty

minutes after TACE, the patient complained of a serious headache and followed by confusion. Non-contrast enhanced CT scanning showed no hyper-intense lesions in the bilateral lungs, but multiple disseminated hyper-intense lesions in the brain, consistent with the deposition of lipiodol. His neurologic symptoms disappeared completely when discharged 9 d later.

DISCUSSION

TACE can result in various severe complications, including acute hepatic failure, intrahepatic biloma, pseudoaneurysm formation, and ectopic infarction, which occur in less than 1% of the patients. Although CLE is a rare complication of TACE, sporadic cases of CLE after TACE have been reported[1, 2]. We encountered a case of CLE after TACE, which was probably associated with hepatic or right inferior phrenic arteriopulmonary vein shunt. The patient had no specific respiratory symptoms such as cough, dyspnoea, but had neurological symptoms including headache and followed by confusion. CT scanning showed some positive findings, indicating deposition of lipiodol, and the diagnosis of CLE was confirmed clinically.

The underlying mechanisms of CLE after TACE are still obscure. Hepatic arterio-pulmonary vein shunt, which is associated with pulmonary vein invasion of HCC, may be the reasonable explanation for CLE. Vascular abnormalities, referred to as hepatic vein invasion, pulmonary arteriovenous shunt, can be found in patients with advanced HCC[3]. An intracardiac right-to-left shunt *via* a patent foramen ovale or intrapulmonary arteriovenous shunt can lead to CLE. Patients with advanced HCC are likely to have a pulmonary arteriovenous shunt[4], and a right-to-left shunt from the RIPA to the pulmonary vasculature is also a possible route[5]. It has been shown that fat globules < 7 μm in diameter can pass directly through the pulmonary arteriolar network (i.e., transpulmonary shunt) and result in cerebral injury[6]. Therefore, presence of intracardiac shunt may not be a requisite for CLE as has been demonstrated in mongrel dogs[7]. But this kind of patients usually had specific respiratory symptoms such as cough, dyspnoea and so on. Wu *et al*[1] thought that pulmonary and CLE might be correlated closely with the bypass between tumor feeding artery and pulmonary vessels due to the tumor invading the thoracic cavity. Matsumoto *et al*[2] concluded that communication between tumor feeding artery and pulmonary vein might have occurred *via* adhesive pleural or tumor invasion into the diaphragm. Therefore, a small dose of lipiodol could enter into the systemic circulation quickly and caused CLE. Thus, we hypothesize that lipiodol passed through the hepatic or right inferior phrenic arteriopulmonary vein shunt or hepatic arteriovenous shunt, and then traveled to the cerebral artery through intrapulmonary arteriovenous shunt.

Although CLE is a rare complication of TACE in patients with HCC, we should keep alert when we observe complications of TACE. When angiogram shows any hepatic arteriovenous or hepatic arteriopulmonary vein shunts, we should decrease the dose of lipiodol during the procedure and pay attention to the respiratory and neurological symptoms after the procedure, which may be caused by ectopic embolism.

In addition to after-embolization syndrome, with its symptoms manifested as fever, pain, nausea and vomiting, there are also some severe complications of TACE, including acute hepatic failure, intrahepatic biloma, pseudoaneurysm formation, ectopic infarction, *etc*. Clinicians should keep in mind that a small number of patients after TACE will suffer from some severe and rare complications. Our patient developed a severe headache and followed by confusion 20 min after the procedure. If some symptoms such as cough, chest pain, chest distress, headache, nausea, and vomiting occur in patients after TACE, physical examinations should be done so as to exclude pulmonary and cerebral complications.

In conclusion, even though CLE is an uncommon complication of TACE, we should be aware of the rare complications in patients with high-risk factors such as a large-size tumor, hepatic vein or diaphragm invasion of tumor and congenital cardiovascular disease, and reduce the dose of lipiodol or stop the procedure accordingly. To reduce the risk of lipiodol embolism, a small lipiodol dose and detection of intracardiac shunt before TACE should be considered in the HCC patients with high risk factors.

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World Journal of Gastroenterology

MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual
Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

World Journal of Gastroenterology

INSTRUCTIONS TO AUTHORS
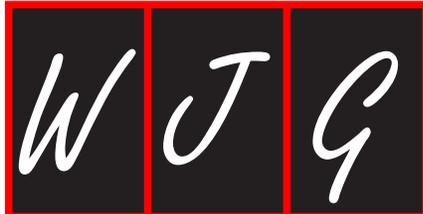
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Organization as author

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5   **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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15   Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16   **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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