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[2]Passed away on June 11, 2007
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EDITORIAL

Gastric carcinoids: Between underestimation and overtreatment

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Abstract

Gastric carcinoids (GCs), which originate from gastric enterochromaffin-like (ECL) mucosal cells and account for 2.4% of all carcinoids, are found increasingly in the course of upper gastrointestinal tract endoscopy. Current nosography includes those occurring in chronic conditions with hypergastrinemia, as the type 1 associated with chronic atrophic gastritis, and the type 2 associated with Zollinger-Ellison syndrome in multiple endocrine neoplasia type 1, and type 3, which is unrelated to hypergastrinemia and is frequently malignant, with distant metastases. The optimal clinical approach to GCs remains to be elucidated, depending upon type, size and number of carcinoids. While there is agreement concerning the treatment of type 3 carcinoids, for types 1 and 2, current possibilities include simple surveillance, endoscopic polypectomy, surgical excision, associated or not with surgical antrectomy, or total gastrectomy. Moreover, the recent introduction of somatostatin analogues represents a therapeutic option of possibly outstanding relevance.

Key words: Gastric carcinoids; Endocrine tumors; Well-differentiated tumors; Hypergastrinemia; Chronic atrophic gastritis; Zollinger-Ellison syndrome; Multiple endocrine neoplasia tupe 1; Enterochromaffin-like cells

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INTRODUCTION

The term gastric carcinoid (GC) describes inadequately the pathological continuum of a wide spectrum of distinct neoplasms that arise from gastric enterochromaffin-like (ECL) cells. Carcinoid tumors represent a variety of significantly diverse lesions, which are distinct from adenocarcinomas in their etiology, biological behavior and prognosis. Over the past 5 years, a marked increase in reports addressing GCs has been evident[1]. These tumors are also known by their modern term of gastric neuroendocrine tumors, although the term carcinoid is still commonly used. This review focuses on the biology, diagnosis and treatment of GCs.

EPIDEMIOLOGY

GC tumors that arise from ECL cells have long been considered as rare lesions, and account for less than 2% of all carcinoids tumors and less than 1% of all stomach neoplasms[1-3]. However, recent reviews have indicated that the incidence of GCs may be on the rise[4-6]. In fact, a recent analysis[4] of the National Cancer Institute's (NCI) Surveillance, Epidemiology, and End Results (SEER) database by Modlin *et al* found that, from 1992 to 1999, GCs comprised 8.7% of all gastrointestinal carcinoid tumors. Also, during the period 1950-1999, a total of 562 GCs were recorded in the NCI databases, but from 2000 to 2004, in the SEER database, 1043 new GCs have been reported, which comprises 11.7% of all gastrointestinal carcinoid tumors[7]. On the other hand, a major decline in incidence and mortality of gastric adenocarcinomas has been described over several decades[8]. The male:female ratio for GCs is about 1:2, with 64% of carcinoids found in women, whereas males are almost twice as likely to develop non-carcinoid

Table 1 Characteristics of GC types

	Type 1	Type 2	Type 3
Percentage (%)	70-85	5-10	15-25
Tumor characteristics	Often small, multiple, polypoid, multicentric	Often small, multiple, polypoid, multicentric	Single, > 1-2 cm, polypoid and often ulcerated
Mean age at diagnosis (yr)	63	50	55
Gender	Females > males	Females = males	Males > females
Associated conditions	Chronic atrophic gastritis type A	ZES/MEN1	Sporadic
Serum gastrin levels	Increased	Increased	Normal
pH of gastric juice	Increased	Low	Normal
Ki-67 (%)	Usually < 2	Usually < 2	Usually > 2
Metastases (%)	2-5	< 10	> 50

gastric-cancer (ratio male:female 1.71)[3].

The reasons for the recent marked increase in GCs are unknown, although the wide use of screening upper endoscopy, the routine habit to obtain biopsies in the course of upper gastrointestinal endoscopy, the application of specific immunohistological identification techniques, and a greater clinical focus on the subject may contribute to increased detection of GCs[9]. On the other hand, our knowledge on the biological basis of these tumors, as well as on the complex interplay between genetic and environmental factors that ultimately results in GC development, are still partial. Hypergastrinemia represents a necessary condition for the development of type 1 and type 2 GCs, even if not sufficient[5,10]. The widespread use of proton pump inhibitors can also induce gastric achlorhydria, thus contributing to hypergastrinemia[11,12], even if it is not clear that it has a real association with an increased risk of GCs. On the other hand, the importance of genetic and molecular background remains to be elucidated. Loss of heterozygosity at the multiple endocrine neoplasia type 1 (MEN-1) gene locus 11q13 has been found in all type 2 tumors that are associated with Zollinger-Ellison syndrome/MEN-1, but also in 17%-73% of type 1, and in 25%-50% of type 3 GCs, although these tumors do no develop in MEN-1 patients[13]. A role for the apoptosis-inhibiting protein BCL-2 has also been proposed, with the hypothesis that the anti-apoptotic activity of BCL-2 may contribute to the development of carcinoid tumors by extending the exposure of hyperplastic ECL cells to other so-far-unknown oncogenic factors[14]. Mcl-1 protein expression also increased specifically in human hypergastrinemia-associated type 1 GC tumors. Gastrin-induced mcl-1 expression may therefore be an important mechanism that contributes toward type 1 GC development[15].

CLASSIFICATION

GCs are endocrine tumors of the gastric mucosa that originate from ECL cells[12,13,16-20]. These tumors are classified into three distinct types (Table 1).

Type 1 (GC-1) includes the vast majority (70%-85%) of GCs and is closely linked to chronic atrophic gastritis type A, characterized by decrease acidity, resultant hypergastrinemia and subsequent ECL cell hyperplasia. The spectrum of ECL cell lesions includes hyperplasia (simple, linear and micronodular), dysplasia, and eventually, carcinoids[21]. The lesions are located in the gastric fundus and body and are multicentric, polypoid, small, limited to the mucosa or submucosa, without angioinvasion, well-differentiated, and tend to display benign behavior. It is more frequent in females.

Type 2 (GC-2) accounts for 5%-10% of GCs, is associated with ZES and occurs almost exclusively in the context of MEN-1. MEN-1/ZES patients usually have small duodenal or pancreatic gastrinomas causing hypergastrinemia and subsequent ECL proliferation. The increased incidence of GC-2 in patients with MEN-1 (13%-37%), who display loss of heterozygosity at the MEN-1 gene locus, versus patients with sporadic ZES (0%-2%), supports the genetic role in the pathogenesis of GCs. Type 2 GCs are usually multiple and small, and have low-grade malignancy, although up to 35% of cases are metastatic at presentation. Unlike GC-1, GC-2 is equally frequent in male and female patients[22,23].

Type 1 and type 2 GCs are both associated with hypergastrinemia. In the first case, hypergastrinemia is secondary to hypo/achlorhydria caused by the destruction of gastric parietal cells. In the second case, it is caused by the presence of a primary gastrinoma that, on the contrary, causes hyperchlorhydria. Therefore pH of gastric juice and blood test are useful to discriminate the presence of pernicious anemia by ZES/MEN1. Pernicious anemia is characterized by increased gastric juice pH, low vitamin B12 and presence anti-parietal cells and/or anti intrinsic factor antibodies. The presence of ZES/MEN1 is characterized by low gastric juice pH or better by a basal acid output $\geqslant$ 15 mEq/h. This condition can be investigated by testing a full evaluation of pituitary and parathyroid function, in addition to genetic analysis.

Type 3 (GC-3) represents 15%-25% of GCs, is not related to hypergastrinemia, is characterized by a far more aggressive course, and presents with lymph node and distant metastases in more than 50% of cases. Lesions are typically solitary, larger than 1-2 cm, ulcerated and deeply invasive. They are usually located in the gastric fundus and body, but may occur also in the antrum. This type of GC is more frequent in males[1,3,12,17,18]. Unlike GC-1 and GC-2, GC-3 may be associated with an atypical carcinoid syndrome that

Table 2 Clinicopathological characteristics of endocrine tumors of the stomach according to WHO classification[23]

Well-differentiated tumor-carcinoid
Benign behavior: confined to mucosa-submucosa, non-angioinvasive, ≤ 1 cm in size, non-functioning
ECL cell tumor of corpus-fundus associated with hypergastrinemia and chronic atrophic gastritits (CAG) or MEN1 syndrome
Serotonin-producing tumor
Gastrin-producing tumor
Uncertain behavior: confined to mucosa-submucosa, > 1 cm in size, or angioinvasive
ECL cell tumor with CAG or MEN1 syndrome or sporadic
Serotonin-producing tumor
Gastrin-producing tumor
Well-differentiated endocrine carcinoma-malignant carcinoid
Low-grade malignant, deeply invasive (muscularis propria or beyond), or with metastasis
Nonfunctioning
ECL cell carcinoid, usually sporadic, rarely in CAG or MEN1 syndrome
Serotonin-producing tumor
Gastrin-producing tumor
Functioning
ECL cell carcinoid with atypical carcinoid syndrome
Serotonin-producing carcinoid with syndrome
Gastrin-producing carcinoma-malignant gastrinoma
ACTH-producing carcinoma with Cushing syndrome
Poorly differentiate endocrine carcinoma-small cell carcinoma, high grade malignant, usually non-functioning, occasionally with Cushing syndrome

Table 3 Proposed TNM staging system for GC tumors[7]

	Primary tumor		
	Depth of invasion		Size
T1	Up to and including muscularis propria		≤ 3 cm
T2	Beyond muscularis propria		≤ 3 cm
T3	Up to and including muscularis propria		> 3 cm
	Beyond muscularis propria		> 3 cm
	Lymph node		
N0	No lymph node metastasis		
N1	Regional lymph node metastasis		
	Distant metastasis		
M0	No distant metastasis		
M1	Distant metastasis		
Disease stage	T	N	M
Ⅰ	T1	Any N	M0
Ⅱ	T2	N0	M0
	T3	N0	M0
Ⅲ	T2	N1	M0
Ⅳ	T3	N1	M0
	Any T	Any N	M1

presents with itching, bronchospasm and cutaneous flushing, thought to be mediated by histamine released from ECL cells[1].

Also, type 4 GCs (GC-4) have been described[17]. This type of tumor is not derived from ECL cells, but from other endocrine cells of the stomach, such as those producing serotonin or gastrin. These tumors may have a very aggressive course and may be located in the gastric fundus, body or antrum.

According to the WHO classification[24], type 1 GCs are well-differentiated endocrine tumors with a benign or, more rarely, an uncertain behavior. Type 2 GCs are usually well-differentiated endocrine tumors, but may also be well-differentiated endocrine carcinomas with angioinvasion, invasion of muscularis propria, and metastases at regional lymph nodes, or less frequently at distant sites. Also, occasionally poorly differentiated endocrine carcinomas have been found in patients with ZES/MEN-1. Type 3 CGs may be well-differentiated endocrine tumors or carcinomas, but usually are poorly differentiated endocrine carcinomas with high mitosis rates and Ki-67 values, and regional and distant metastases (Table 2). Moreover, recently, a tumor-node-metastasis (TNM) staging, and a grading system, based on the proliferative status (mitotic count and Ki-67 index) have been suggested for GCs[7] (Table 3), but remain to be validated in clinical practice.

DIAGNOSIS

Diagnosis is currently made during upper gastrointestinal endoscopy performed for a variety of clinical reasons, such as abdominal pain, gastrointestinal bleeding, anemia and dyspepsia. The diagnostic accuracy and the correct characterization of GCs necessitate extensive sampling from both the antrum (two samples) and body-fundus (four samples), in addition to biopsies/removal of the largest polyps. Proliferation rate and degree of dysplasia of gastric endocrine cells may often be difficult to identify with standard histopathological procedures. Histochemistry with chromogranin A (CgA) and synaptophysin assessment is of relevance in identifying hyperplasia, dysplasia and malignant transformation of ECL cells[20,25,26]. Also, immunohistochemical determination of the proliferative index Ki-67 and evaluation of the mitotic index, by counting number of mitosis per 10 high-power fields, are mandatory[27], with a negative prognostic meaning when Ki-67 is > 2% and mitotic index is > 2.

Endoscopy and sampling for histology are currently

considered sufficient when faced with small type 1 and type 2 GCs, reserving endoscopic ultrasound (EUS) for tumors > 1 cm in size[27]. EUS can give information about the location and depth of lesions and local spread, or even highlight the primary gastrinoma in GC-2. EUS can also allow fine-needle aspiration of submucosal lesions.

Computed tomography, magnetic resonance imaging and somatostatin receptor scintigraphy are required for larger tumors, those shown to be invasive by EUS, and type 3 GC, in order to detect distant metastases[27]. The minimal biochemical tests in GC patients include serum gastrin and CgA levels, the most important generic marker for neuroendocrine tumors, with evaluation of gastric juice pH. These tests should be performed at diagnosis. Moreover, determination of CgA could be of relevance in the course of follow-up[5,21,27].

PROGNOSIS

GCs are usually considered as largely benign in prognosis, even if it depends on the type of GC tumor and the extent of the disease. Prognosis ranges from an indolent course for type 1 GCs to the worst one for type 3 GCs.

Rappel and colleagues[28] reported an overall survival rate of 78% in 110 patients with GCs, with the highest rate (100%), when aged-corrected, in the 88 patients with GC-1. Therefore, the authors concluded that patients with GC-1 tumors have a life expectancy comparable to that of the general population. Type 2 GCs have a similar outcome to type 1 GCs, although their overall survival is closely related to the course of the associated gastrinoma, with a 5-year survival of 62%-75%[29]. Type 3 GCs have the worst prognosis and are typically associated with an overall 5-year survival of < 50%[2]. On the other hand, in an update of the SEER database study by Modlin *et al*[4], the 5-year survival rate was 63% for all GCs, 21.2% for metastatic disease, and only 69.1% in the subset of patients with localized lesions. Moreover, a cumulative analysis of GCs in the SEER database from 1992 to 1999 has indicated that distant metastases or regional spread were evident in 10%-30% of cases at the time of diagnosis, thus suggesting that the widespread opinion regarding the benign behavior of GC tumors should be revised.

A further frustrating finding is represented by the lack in the last 30 years of changes in mean overall survival for patients with GCs, as well as for those with other gastroenteropancreatic neuroendocrine tumors[9,30], despite the increased proportion of patients diagnosed at an earlier stage of the disease. However, it should be noted that many variables, other than types of GC, can affect the overall prognosis, such as age, gender, ethnicity, tumor size, depth of invasion, lymph node involvement, distant metastasis, degree of differentiation, and histological subtype.

MANAGEMENT

The clinical approach to GCs is largely dependent upon the type and size of lesions (Figure 1). Management of type 3 GC is fairly clear and comparable to that used for gastric adenocarcinomas, which includes partial or total gastrectomy with extended lymph node resection[1,3,12,16] in the absence of visceral metastases, or systemic chemotherapy if surgery is not feasible, even if, so far, the results are not very encouraging. The questionable efficacy of conventional cytotoxic chemotherapy has prompted investigation of novel therapeutic approaches for patients with advanced carcinoid. These include the use of targeted radiotherapy, as well as regimens incorporating inhibitors of angiogenesis (e.g. bevacizumab) and small molecule tyrosine kinase inhibitors (e.g. sunitinib). The treatment of metastatic liver disease includes hepatic resection, embolization of the hepatic artery, radiofrequency ablation and cryoablation[31].

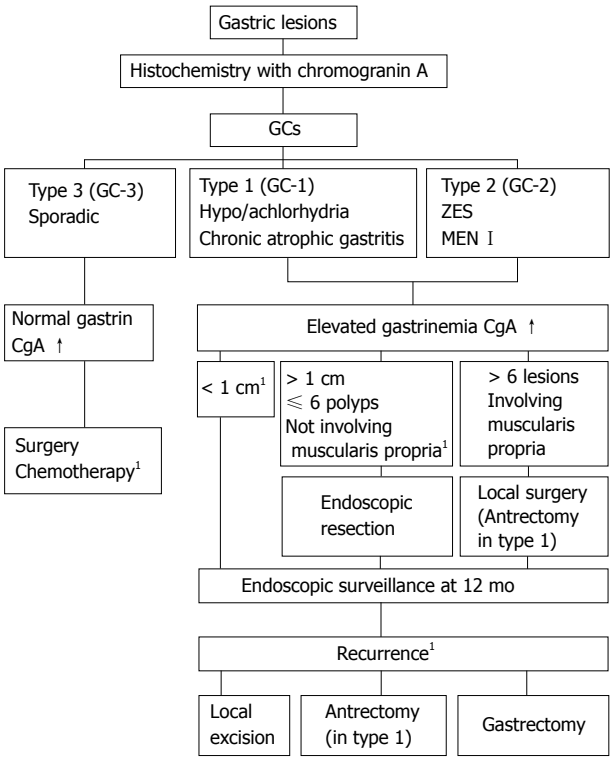

Figure 1 Management flow chart of GCs according to ENETS guidelines[26]. [1]Consider SSAs.

We consider the more controversial management of types 1 and 2 GCs, which are characterized by more benign biological behavior. In GC-1, a conservative approach based on endoscopic resection seems to be the treatment of choice when the size (< 1 cm) and the number (< 3-5) of the tumors render it feasible[1]. However, recently the European Neuroendocrine Tumor Society (ENETS) Consensus Guidelines[27] have suggested that annual surveillance is appropriate when dealing with patients with type 1 GC of less than 10 mm in size. This practical approach is supported by some reports[28,32,33] that suggest that the above careful endoscopic follow-up represents a reasonable and safe option in selected patients. However, further studies including a more consistent number of patients and

with an adequately long follow-up are necessary to support this statement. In fact, despite their usually benign biological and clinical course, type 1 GCs can sometimes exhibit a not entirely negligible mortality rate, as deducible from series with long follow-up[4]. In case of tumors > 10 mm and with up to six polyps not involving the muscularis propria at EUS examination, endoscopic resection remains the reference approach[27]. In the presence of deep gastric parietal wall invasion and positive margins following endoscopic mucosal resection, surgical resection of the tumor should be carried out[27]. Once again, it should be noted that, with these tumors often being multiple and recurrent, antral resection, aimed at avoiding chronic ECL cell stimulation by ongoing hypergastrinemia, is recommended, which is effective in 80% of type 1 tumors[27,34-36]. Moreover, in the case of malignant transformation or recurrence despite local surgical resection, partial or total gastrectomy with lymph node dissection should be performed, as suggested by current guidelines[27].

Overall, despite a generally benign prognosis, the recommended approach in selected subgroups of GC-1 patients appears disproportionately aggressive, and the long-term benefits of antrectomy are still uncertain[34]. Indeed, in some cases, the tumors may become autonomous and no longer gastrin-dependent, and therefore, continue to grow after antrectomy. An octreotide suppression test has been proposed[37] to predict the beneficial outcome from antrectomy, by measuring histidine decarboxylase (HDC) mRNA in the pre- and post-treatment biopsy specimens. In fact, HDC is the enzyme that catalyzes the synthesis of histamine from histidine in ECL cells, a process that is gastrin dependent. A marked decrease in HDC mRNA after octreotide administration indicates that the tumor is still likely to be gastrin dependent.

In extreme situations, i.e., when the biological behavior of the tumor is well defined and definitely benign or malignant, the current guidelines are clear and unambiguous. Conversely, they are less clear for GCs with uncertain behavior, which show atypical characteristics, such as elevated Ki-67, or submucosal invasion, even if they are smaller than 1 cm. Moreover in this situation, according to current guidelines, only endoscopic follow-up is indicated, therefore, information about deep invasion and margin infiltration is not available. At present, relevant controversies and doubts remain in these particular subgroups of patients. It should be stressed that the overall approach is based mainly on the tumor size, but this parameter may not represent the only prognostic factor. Recent studies[38,39] have suggested that proliferation indexes such as Ki-67 are of relevance, but the current best aggregate indicators of prognosis and malignancy seem to be the evidence of invasive growth and the presence of regional or distant metastases (TNM staging system)[38]. At present, however, the criteria to delineate the degree of malignancy remain unclear, and the histological analysis often fails to define precisely the likelihood of aggressive or metastatic potential.

Over the last few years, somatostatin analogues (SSAs) have been used in the treatment of patients with either GC-1 or GC-2[40-45], based on their capability to inhibit gastrin release from the antral G cells, thus reducing ECL cell hyperplasia. However, biotherapy is not currently recommended in patients with type 1 and 2 tumors, except in the rare patients with functioning tumors, and in type 2 patients if indicated for an underlying disease (i.e., other endocrine tumors). Preliminary reports[41] have shown that SSAs have some beneficial effects, for example, by reducing the size and number of carcinoids tumors after 6 mo of treatment. Moreover, the treatment with long-acting SSAs given at monthly intervals for a period of at least 6 mo produces significant suppression in gastrin and CgA levels[40]. Overall, however, the best schedule of treatment remains to be defined.

The management of type 2 GC has to be approached in the context of the MEN-1 syndrome that is present in these patients. As for type 1 GC, endoscopic treatment can be an option, whereas gastric surgery should be performed only in highly selected patients, particularly if the histological examination shows the features of poorly differentiated endocrine tumors. The treatment of type 2 GCs is further complicated by the controversies regarding the treatment of gastrinoma in MEN-1. Currently, no definitive evidence exists that surgery decreases the mortality in MEN-1 or the likelihood that clinically important metastases will develop. Then, the question of whether or not to recommend duodenal-pancreatic surgery in patients with MEN-1 who have pharmacologically controllable ZES and no other clinically evident hormonal excess syndrome is a difficult one. In these cases, the SSA octreotide has been demonstrated to be effective at reducing tumor growth[43].

CONCLUSION

A lot of controversies still exist about the optimal treatment of GC tumors. In fact, endoscopic follow-up could have some risk and is expensive, which leads to further examinations. On the other hand, a more aggressive approach, based on endoscopic or surgical resection may represent over-treatment, with possible unnecessary side effects and high costs. Treatment with long-acting SSAs may therefore represent an alternative option that, even if expensive, seems to be both efficient and safe. Based on the current lack of validated recommendations[40,41,44,45], SSAs should probably be reserved for tumors with atypical characteristics or for multiple small tumors, when surgery is not feasible or judged excessive, and when iterative endoscopic removal is too fastidious or impractical.

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EDITORIAL

Signal transduction pathways in liver and the influence of hepatitis C virus infection on their activities

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Abstract

In liver, the most intensively studied transmembrane and intracellular signal transduction pathways are the Janus kinase signal transduction pathway, the mitogen-activated protein kinases signal transduction pathway, the transforming growth factor β signal transduction pathway, the tumor necrosis factor α signal transduction pathway and the recently discovered sphingolipid signal transduction pathway. All of them are activated by many different cytokines and growth factors. They regulate specific cell mechanisms such as hepatocytes proliferation, growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix. The replication cycle of hepatitis C virus (HCV) is intracellular and requires signal transduction to the nucleus to regulate transcription of its genes. Moreover, HCV itself, by its structural and non-structural proteins, could influence the activity of the second signal messengers. Thus, the inhibition of the transmembrane and intracellular signal transduction pathways could be a new therapeutic target in chronic hepatitis C treatment.

Key words: Liver; Hepatitis C virus infection; Signal transduction pathway; Proliferation; Apoptosis

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INTRODUCTION

Hepatitis C virus (HCV) was discovered by Choo *et al*[1]. in 1989. HCV is included in the Flaviviridae family within the distinct genus of *Hepacivirus*[2]. According World Health Organization (WHO) data, there are currently about 170 million HCV-infected persons worldwide, which is approximately 3% of the human population. In Poland, the number of chronic HCV-infected persons is estimated to be 750 000, which is about 1.4 % of the general population[3]. In the natural history of HCV infection, there is an 80% risk of chronic infection, as well as the high possibility of severe complications such as liver cirrhosis or hepatocellular cancer (HCC).

The main target cell for HCV infection is the hepatocyte, however the virus also infects B lymphocytes and affects other immune system components. The HCV replication cycle is intracellular and requires activation of many transmembrane and intracellular signal transduction pathways, which are mainly activated by cytokines such as tumor necrosis factor α (TNF-α), interleukins (IL-4, IL-6, IL-12 or IL-13), interferons, mitogens hepatocyte growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor α (TGF-α) and growth inhibitors (TGF-β and activine).

TRANSMEMBRANE AND INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS

Janus kinase (JAK) signal transduction pathway

The JAK signal transduction pathway is activated by more than fifty different cytokines and growth factors. This intracellular pathway operates not only in hepatocytes but also in immune, hematopoietic and neural system cells. After extracellular ligand-receptor interaction, receptor multimerization and the activation

Table 1 STATs activation and function

STAT protein	Activating factor	Activation effect
STAT 1	IFN-α/β (type Ⅰ INF) IFN-γ (type Ⅱ INF)	Antiviral response, inflammation and hepatocyte damage development, apoptosis stimulation
STAT 2	INF-α/β i INF-λ	Antiviral response
STAT 3	IL-6 and its family, IL-10, IL-22, EGF, HCV proteins	Participates in antiviral IFN-α effect, direct cytoprotective and anti-inflammatory influence on hepatocytes
STAT 4	IL-12	Probably plays a critical role in hepatocytes damage during hepatic ischemia/reperfusion injury and in Th1 differentiation
STAT 5	Growth factors	Regulates the genes expression essential for hepatocytes metabolism, growth and differentiation
STAT 6	IL-4, IL-12 and IL-13	Participates in Th2 lymphocytes response during viral hepatitis and decreases hepatocytes damage during hepatic ischemia/reperfusion injury

of JAK1, JAK2, JAK3 and tyrosine kinase 2 (Tyk2) is observed. The receptor-kinase complex phosphorylates cytoplasmic SH-2-containing transcription factors: signal transducers and activators of transcription (STAT) 1, 2, 3, 4, 5, 6. Activated STATs present two main functions: signal and transcriptional by forming homo- and heterodimers, which translocate to the nucleus to influence transcription. STATs are specifically inhibited by protein inhibitors of activated STAT (PIAS)[4] and by suppressor of cytokine signaling (SOCS) through negative feedback control (Figure 1A). SOCS proteins include SOCS 1, 2, 3 and cytokine-induced Src homology 2 protein (CIS), which bind to JAK kinase inhibiting its enzymatic activity[5].

STATs perform different, often opposing functions in the liver. STAT1 is mainly activated by IFN type I (IFN-α/β) and IFN type Ⅱ (IFN-γ). Its essential function in liver is the participation in antiviral immune defense, as well as in the development of inflammation and apoptosis. IFN-α/β and IFN-λ are ligands for STAT2, whose major function is antiviral defense. Membrane the IFN-α/β receptor (IFNAR) is a complex of two subunits: IFNAR1 and IFNAR2. IFNAR2 presents three diverse forms: full-length IFNAR2c is responsible for signal transduction and transcription process, whereas short form IFNAR2b and soluble form IFNAR2a inhibit these processes[6]. The complex IFN-α/β - IFNAR activates JAK1 and Tyk2 kinases. IFN-γ takes effect by IFN-γ receptor (IFNGR): IFNGR1 and IFNGR2. STAT3 function is especially regulated by IL-6 and its family members such as cardiotrophin-1 (CT-1), oncostatin M (OSM), IL-11, leukemia inhibitory factor (LIF) or ciliary neurotrophic factor (CNTF), by IL-10, IL-22, EGF and HCV proteins. STAT3 participates in the acute phase response, stimulates hepatocytes regeneration and regulates lipid and carbohydrate metabolism in the liver[7]. Moreover STAT3 is one of the main anti-HCV-defense elements that acts by increasing the IFN-α antiviral effect and by its direct cytoprotective and anti-inflammatory influence on hepatocytes[8]. IL-6 and its related cytokines bind gp130 receptor protein, which plays a key role in liver regeneration.

Furthermore, Li *et al*[9] confirmed that gp130 activity is independent of the activities of other kinases, such as MAKP. The ligand-gp130 complex activates JAK1, JAK2 and Tyk2. Recently, the influence of HCV infection on STAT1-3 factors was demonstrated. HCV structural proteins C, E2 and non-structural protein NS5A were shown to reduce the number of membrane receptors (IFNAR1 and IFNAR2c) blocking STAT1-3 activation by IFN-α. STAT1-3 are also inactivated by ethanol and increased level of TNF-α, IL-1β and IL-10[7]. As a result, viral replication, as well as inflammation and fibrosis in the liver, is augmented and has a negative effect on IFN-α treatment response among patients with severe liver damage. However, HCV does not affect IFN-γ function, and in consequence, STAT1 activation[10]. Moreover, Sun and Gao showed that IFN-γ produced by NK cells inhibits hepatocytes regeneration during HCV infection[11]. STAT4 is the least known transcription factor. STAT4 has been shown to be activated by IL-12 and to play a critical role in hepatocytes damage during hepatic ischemia/reperfusion injury and in Th1 differentiation[12]. STAT5 is mainly activated by growth factors and regulates the expression of genes encoding cytochrome P450, HGF and insulin growth factor 1 (IGF1), which are essential for hepatocytes metabolism, growth and differentiation. STAT6 is regulated by IL-4, IL-12 and IL-13. These factors participate in Th2 lymphocytes response during viral hepatitis and reduce hepatocytes damage during hepatic ischemia/reperfusion injury. A summary of STATs activation and function are shown in Table 1.

MAPK signal transduction pathway

EGF, HGF and TGF-α bind with membrane receptors having intrinsic tyrosine kinase enzymatic activity. Ligand-receptor complex multimerization and autophosphorylation are then observed. Ras proteins and GTP create a transient complex activating RAF kinases and MAPK kinases (MKK), which can activate MAPK by dual phosphorylation of threonine and tyrosine. Activated MAPK phosphorylates transcription factors such as cAMP response element-binding (CREB) and Ets-related transcription factor 1 (ELK-1). The MAPK signal transduction pathway is evolutionarily one of the oldest signal transduction pathways in eukaryotic cells. It contains three different signal tracts: the extracellular regulated protein kinase (ERK, p42/44 MAPK) tract, the stress activated protein kinase (SAPK, p38 MAPK, p38-RK or p38) tract, and the c-Jun-NH2-terminal kinase (JNK, p64/54 SAPK) tract (Figure 1B). All of

these pathways regulate processes such as cell growth, differentiation, maturation, proliferation and apoptosis. In mammalian cells every single pathway is activated by two MKK: JNK by MKK4 and MKK7, ERK by MKK1 and MKK2, and SAPK by MKK3 and MKK6. This dual role of MKK in the activation of the JNK, ERK and SAPK signal transduction pathways is still unclear[13]. It has been shown that ERK play a key role in the regeneration of the majority of eukaryotic cells. However the role of SAPK, especially in hepatocytes regeneration, is as yet undefined. Physiologically, the activity of JNK in the liver is minimal but increases during liver regeneration, probably associated with high hepatic TNF-α levels[14].

TGF-β signal transduction pathway

TGF-β is cytokine family member that plays a key role in the processes of cell growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix. In the liver, during HCV infection, TGF-β is responsible for hepatocytes regeneration and fibrosis, and for epithelial cells proliferation and differentiation. TGF-β1 serum concentration in patients with chronic liver diseases, including chronic HCV infection, is higher the more severe the liver failure is, confirming the association between this cytokine and hepatic fibrosis[15]. Concurrently, in patients with chronic hepatitis C, TGF-β1 serum concentration decreases and normalizes after successful antiviral therapy[16].

The TGF-β membrane receptor consists of two subunits having intrinsic serine/threonine kinase enzymatic activity: the type Ⅰ receptor (TβR-Ⅰ) and the type Ⅱ receptor (TβR-Ⅱ). After binding of the ligand to TβR-Ⅱ, TβR-Ⅰ is phosphorylated in the GS domain containing many glycine and serine amino acids. Activated TβR-Ⅰ influences receptor-specific R-Smad proteins (Smads) and common-partner Smad (Co-Smad). SMADs are a class of proteins that modulate the activity of transforming growth factor beta ligands. Newly created complexes translocate to the nucleus and stimulate transcription and apoptosis[17] (Figure 1C). During liver regeneration, elevated TGF-β concentration is observed, though it does not give rise to an increase of hepatocytes apoptosis, which is probably linked with parallel augmentation of the concentrations of Smads: Ski and SnoN, and other antiapoptotic proteins such as Bcl-2 and Bcl-X in hepatocytes[18]. HCV, through the NS5A protein, inhibits TGF-β signal transduction pathway activity. NS5A reacts directly with TβR-Ⅰ using the region between amino acids 148 and 237. As a result, Smads phosphorylation, complex creation and its migration to the nucleus are blocked. In contrast, NS5B protein has no inhibitory effect on TβR-Ⅰ. TGF-β pathway inhibition can be the result not only of HCV infection, but also of other viruses such as hepatitis B virus (HBV), adenoviruses and HPV. This effect can be due to the direct interaction between the X protein and Smad4 (HBV), interaction between the E1 protein and R-Smad (adenoviruses) or through the inhibitory effect of the E7 protein on R-Smad and Co-Smad complex formation in the nucleus (HPV)[19].

TNF-α signal transduction pathway

TNF-α is produced by macrophages, monocytes, mast cells and NK cells. TNF-α is one of the main mediators of the antiviral inflammatory response, which enhances lymphocytes proliferation and differentiation, acute phase proteins production and cell apoptosis. Two essential TNF-α membrane receptors are known: TNF-R1 (CD120a, TNF-55r or p55) and TNF-R2 (CD120b or p75). TNF-R1 plays a key role in the liver due to its presence not only in hepatocytes membrane but also in Kupffer cells and hepatic sinusoidal endothelial cells. TNF-R1 consists of three domains: extracellular, transmembrane and intracellular (known as the death domain (DD). Activated TNF-R1 binds, *via* the DD, to an adaptor protein TNFR-associated protein with death domain (TRADD), which afterwards activates Fas associated death domain (FADD) proteins, TNF-associated factor-2 (TRAF-2) and receptor-interacting protein (RIP). All of these proteins influence different signal transduction pathways. FADD activates caspases 8 and 10 leading to Death-Inducing Signaling Complex (DISC) formation, which regulates apoptosis[20], whereas TRAF-2 and RIP activate two tracts taking part in the anti-apoptotic effect of TNF-α: IκB kinase (IKK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB factor), as well as JNK and ERK from MAPK signal transduction pathway[21] (Figure 1D). NFκB is a transcription factor comprising two subunits: p50 having a molecular weight of 50 kDa and p65 (also known as RelA, v-rel reticuloendotheliosis viral oncogene homolog A or nuclear factor of kappa light polypeptide gene enhancer in B-cells 3) having a molecular weight of 65 kDa. The RelA subunit is mainly responsible for the anti-apoptotic function of NFκB. In the cytoplasm, NFκB, with inhibitor proteins IκBα or IκBβ (IKK), creates the inactive form. TRAF-2/RIP activates IKK, which phosphorylates IκB leading to its subsequent degradation in proteasomes. Activated NFκB translocates to the nucleus where it binds with DNA through a zinc finger motif and stimulates transcription of genes encoding cytokines, acute phase proteins, immunoglobulins and adhesion factors[22]. TNF-α linked with TNF-R1 leads, depending on activated cellular proteins, to cell proliferation or apoptosis. Kato *et al*[23] showed that HCV core protein C and, to a lesser degree, NS4B protein, influence cell proliferation and production of proinflammatory cytokines such as IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, TNF-α and INF-β stimulating three diverse pathways through NFκB, activator protein-1 (AP-1) and serum response element (SRE). AP-1 is a complex of homo- or heterodimers encoded by c-jun and c-fos family genes. Moreover, AP-1 stimulates proliferation dependent on growth factors, oncogenes and inflammatory peptides. SRE regulates the promoters of immediate early (IE) genes such as c-fos and PIP92. MAPK cascade activation phosphorylates Elk-1 factor binding with SRE and serum response factor (SRF)[24]. The thus created complexes affect transcription of genes taking part in cell proliferation.

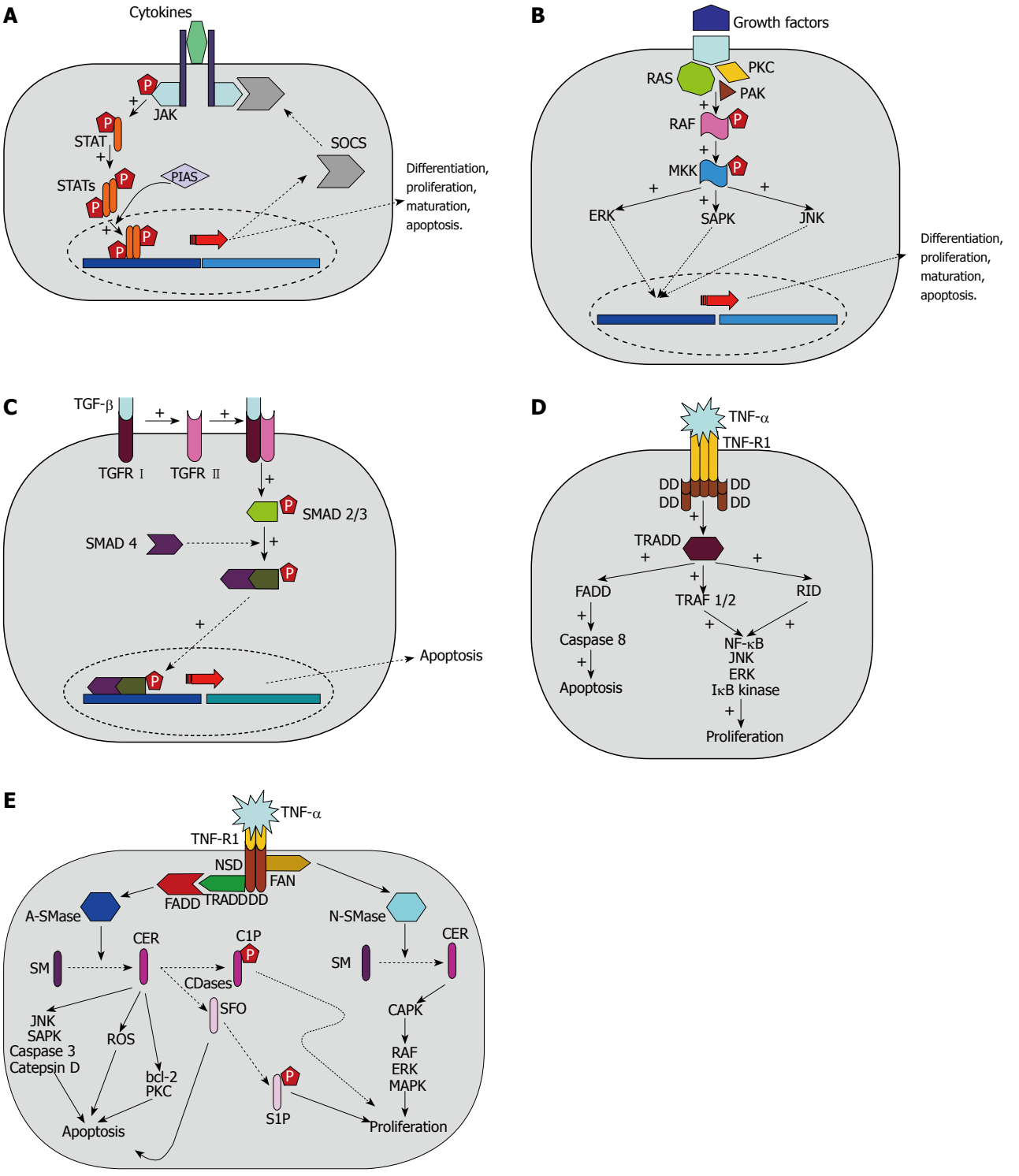

Figure 1 Signal transduction pathway. A: Janus kinase (JAK); B: Mitogen-activated protein kinase (MAPK); C: Transforming growth factor-β (TGF-β); D: Tumor necrosis factor α (TNF-α); E: Sphingolipid. +: Activation; P: Phosphorylation; STAT: Signal transducers and activators of transcription; STATs: Activated STAT; PIAS: Proteins inhibitor of activated STAT; SOCS: Suppressor of cytokine signaling; RAS: Small GTP-binding protein; PKC: Protein kinase C; PAK: P21-activated kinase; RAF: Serine/threonine kinase; MKK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated protein kinase; SAPK: Stress activated protein kinase; JNK: C-Jun-NH2-terminal kinase; TGFR Ⅰ and Ⅱ: Membrane receptors of TGF-β; SMAD: Class of proteins that modulate the activity of transforming growth factor β ligands; TNF-R1: Membrane receptor of TNF-α; DD: Death domain; TRADD: TNFR associated protein with death domain; FADD: Fas associated death domain; TRAF 1/2: TNF-associated factor-2; RID:Receptor-interacting protein; NFκB: Transcription factor; NSD: TNF-R1 domain activating neutral sphingomyelinase; FAN: TNF-R1 adaptor protein; A-SMase: Acid sphingomyelinase; N-SMase: Neutral sphingomyelinase; SM: Sphingomyelin; CER: Ceramide; C1P: Ceramide-1-phosphate; CDases: Ceramidases; SFO: Sphingosine; S1P: Sphingosine-1-phosphate; ROS: Reactive oxygen species; CAPK: Ceramide-activated kinase.

Sphingolipid signal transduction pathway

Initially sphingolipids were demonstrated to be major components of eukaryotic plasma membranes and mediators of cell-to-cell interactions. Since 1989, many studies have shown that sphingolipids are also the essential second messengers in transmembrane and intracellular signal transduction. This new pathway was called the sphingolipid signal transduction pathway[25]. Generally,

it mediates specific cell reactions such as proliferation, growth arrest, differentiation, apoptosis and calcium homeostasis. It is activated by many proapoptotic and promitotic factors, such as cytokines TNFα and IL-1, Fas (Apo-1, CD95) receptor agonists, CD-40, CD-28, CD-5, DR-5, lymphocyte function-associated antigen-1 (LFA-1), CD-32 (FcγRII), CD-20, hormones (progesterone), vitamin D3, protein kinase C inhibitors, growth factors [platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF)], infection (by *P. aeruginosa, S. aureus, N. gonorrhoeae, Sindbis virus* and *Rhinoviru)*, γ radiation, UV and chemotherapeutics (such as doxorubicine and cisplatin)[26]. The final effect of pathway activation (cell survival or death) depends on the inductive factor and the balance between the intracellular levels of its main components: ceramide (Cer) and sphingosine-1-phosphate (S1P). This balance is known as "the Cer/S1P rheostat".

The most intensively studied second messenger of sphingolipid signal transduction pathway is ceramide, which is highly antiproliferative (Figure 1E). Firstly, Cer activates c-Jun kinase (JNK), stress activated protein kinases (SAPK), cathepsin D, methionine adenosyl transferase 1A (MAT1A) and caspase 3, which are responsible for destruction of the cytoskeleton, nuclear and plasma membranes[27]. Secondly, Cer stimulates the mitochondria to release reactive oxygen species (ROS) and cytochrome c, activating the apoptotic proteases[28]. Finally, Cer decreases, by dephosphorylation, the intracellular level of anti-apoptotic proteins of the Bcl-2 family and the activity of anti-apoptotic enzymes like kinases that depend on the intracellular Ca^{2+} levels [protein kinase C, (PKC), PKCα and PKCβa/Akt]. Paradoxically, Cer synthesized from the hydrolysis of sphingomyelin (SM) by neutral sphingomyelinases (NSMases), enhances the activity of the ceramide activated protein kinase (CAPK) and afterwards the serine/threonine kinase Raf and Akt, extracellular signal-regulated protein kinases (ERK 1/2) and the mitogen-activated protein kinase (MAPK). All these kinases stimulate the proliferation process[29]. Cer regulates the cell growth processes through its influence on PKC, kinase suppressor of Ras (KSR), Raf-1, MAPK and ceramide-activated protein phosphatase (CAPP), controlling the protein phosphatases PP1 and PP2. Cer also take a part in plasma membrane reorganization, facilitating transmembrane proapoptotic signal transduction and modulating the autophagocytosis[30]. Autophagocytosis relies on degradation of damaged, dead or used cell structures to prolong cell life. Cer inhibits autophagocytosis by stimulating apoptosis[31].

A further second messenger of sphingolipid signal transduction pathway is sphingosine (SFO). SFO is synthesized from the hydrolysis of Cer by ceramidases (CDases). SFO has a key role in apoptosis by stimulating ROS production in mitochondria and activation of caspase 3, 7 and 8[32]. Additionally, sphingosine inhibits Akt, resulting in the augmentation of the cellular effects of cytochrome c and caspase 3[33]. Moreover, SFO directly blocks DNA synthesis, methylation and replication. SFO also reduces the activity of protein kinases such as PKC, calmodulin-dependent protein kinase and insulin receptor kinase. The PKC inhibition proceeds in two parallel ways: directly and indirectly by decreasing the level of intracellular diacylglycerol (DAG) and Ca^{2+} ions. The PKC inhibition leads to disturbances of nuclear proteins phosphorylation (RNA polymerase, topoisomerase Ⅱ, histones and matrix proteins[34]). Some studies underline the proliferative character of SFO. It seems that low cellular concentrations of SFO stimulate cell proliferation and DNA synthesis, whereas the high concentrations stimulate apoptosis.

Sphingosine-1-phosphate (S1P), synthesized from SFO, has a potent anti-apoptotic character. An increase in the intracellular level of S1P can activate cell proliferation and its passing from G_1 phase to S phase, augment the general number of cells resting in S phase, shorten the time needed for cell division, enhance survival rate of cells subjected to proapoptotic factors, mobilize calcium ions from intracellular compartments, influence cytoskeletal architecture and the processes of cell migration and adhesion. S1P modulates cell functions in two different ways: as an intracellular messenger and as a ligand of G protein-coupled receptors, known as endothelial differentiation genes (Edg) - Edg-1, -3, -5, -6 and -8[35].

Cer may be phosphorylated by ceramide kinase to ceramide-1-phosphate (C1P), which can be dephosphorylated back to ceramide by C1P phosphatase. Similarly to S1P, C1P promotes cell proliferation[36].

Recently, some studies have shown that the inhibition of sphingolipid metabolism can be a new therapeutic target for HCV infection[37].

CONCLUSION

All phases of HCV replication cycle are intracellular and consequently require signal transduction to the host cell nucleus to regulate transcription of viral genes. Although the pathogenesis of transmembrane and intracellular signal transduction during HCV infection is still unclear, it has been shown that HCV could influence activity of the second signal messengers. This mechanism can regulate specific cell mechanisms such as hepatocytes proliferation, growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix, leading to severe complications of chronic HCV infection such as liver cirrhosis or hepatocellular cancer. For instance HCV, through the NS5A protein, inhibits the TGF-β, signal transduction pathway activity and through the core protein C and, to a lesser degree, the NS4B protein, influences production of proinflammatory cytokines such as TNF-α. Accordingly, it seems that the inhibition of the activity of the intracellular messengers and pathways could be a new therapeutic target for chronic hepatitis C treatment, leading not only to overall HCV elimination from hepatocytes and from other extrahepatic components, but also to decrease the possibility of developing chronic hepatitis C complications. Moreover, the discovery of the role of the JAK signal transduction pathway as the principal signaling pathway for IFN-α opens new research options for a better understanding of IFN-α resistance. HCV structural proteins C and E2 and non-structural protein NS5A have been shown to

reduce the number of membrane receptors IFNAR1 and IFNAR2c blocking STAT1-3 activation by IFN-α. As a result, viral replication, as well as inflammation and fibrosis in the liver, are augmented and has a negative effect on IFN-α treatment response among patients with severe liver damage. Therefore, a better understanding of these signaling defects might lead to new therapeutic strategies, making IFN-α therapy more effective in a larger percentage of patients with chronic hepatitis C infection.

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TOPIC HIGHLIGHT

Kostas Pantopoulos, Associate Professor, *Series Editor*

Non-invasive assessment of liver fibrosis in chronic liver diseases: Implementation in clinical practice and decisional algorithms

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Abstract

Chronic hepatitis B and C together with alcoholic and non-alcoholic fatty liver diseases represent the major causes of progressive liver disease that can eventually evolve into cirrhosis and its end-stage complications, including decompensation, bleeding and liver cancer. Formation and accumulation of fibrosis in the liver is the common pathway that leads to an evolutive liver disease. Precise definition of liver fibrosis stage is essential for management of the patient in clinical practice since the presence of bridging fibrosis represents a strong indication for antiviral therapy for chronic viral hepatitis, while cirrhosis requires a specific follow-up including screening for esophageal varices and hepatocellular carcinoma. Liver biopsy has always represented the standard of reference for assessment of hepatic fibrosis but it has some limitations being invasive, costly and prone to sampling errors. Recently, blood markers and instrumental methods have been proposed for the non-invasive assessment of liver fibrosis. However, there are still some doubts as to their implementation in clinical practice and a real consensus on how and when to use them is not still available. This is due to an unsatisfactory accuracy for some of them, and to an incomplete validation for others. Some studies suggest that performance of non-invasive methods for liver fibrosis assessment may increase when they are combined. Combination algorithms of non-invasive methods for assessing liver fibrosis may represent a rational and reliable approach to implement non-invasive assessment of liver fibrosis in clinical practice and to reduce rather than abolish liver biopsies.

Key words: Chronic liver diseases; Hepatic fibrosis; Liver biopsy; Non-invasive methods for liver fibrosis assessment; Combination algorithms; Decisional tree

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INTRODUCTION

Chronic liver diseases (CLDs) represent a major cause of morbidity and mortality worldwide. The major etiologies are chronic infection with hepatitis B (HBV) and C (HCV) viruses, and alcoholic and non-alcoholic fatty liver disease. Chronic hepatitis B and C are the leading causes of cirrhosis and of hepatocellular carcinoma (HCC) worldwide. Approximately 400 million people are chronically infected with HBV and 25%-40% of them die of cirrhosis and of its end-stage complications[1]. HBV is the most important carcinogen after tobacco and the incidence of HCC is 300 000 cases per year[2]. Chronic hepatitis C is a major health concern with around 200 million individuals affected worldwide, with a greater prevalence in Western countries[3]. Natural history studies indicate that advanced fibrosis and cirrhosis develop in about 20%-40% of patients with chronic viral hepatitis[4,5]. Alcoholic liver disease (ALD) is one of the leading causes of end-stage CLD. It is well established that only a minority of heavy drinkers, estimated at between 10% and 30%, will ever develop advanced ALD and that the risk increases with cumulative alcohol intake[6,7]. Non-

alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease and impaired liver function in industrialized countries, where 10%-23% of the adult population is estimated to be affected[8,9]. The disease has a spectrum ranging from fatty liver alone to non-alcoholic steatohepatitis (NASH), and progressive steatofibrosis. Many cases of cryptogenic cirrhosis may be end-stage forms of NASH[10]. Hepatic steatosis is currently considered a manifestation of metabolic syndrome[11,12], which is defined as an association of at least 3 of the following disturbances: insulin resistance, central obesity, arterial hypertension, and dyslipidemia, whether hypertriglyceridemia or low HDL-cholesterol levels. Only a percentage of individuals with liver steatosis progress to more advanced stages of the disease[8-10]. The pathogenesis of NAFLD and the reasons why some patients with fatty liver develop NASH and have progressive liver disease are not entirely understood. The most widely supported theory implicates insulin resistance as the key mechanism in NAFLD, leading to hepatic steatosis, and perhaps also to NASH. Obesity, type 2 diabetes, hyperlipidemia and other conditions associated with insulin resistance are generally present in patients with NAFLD[11,12]. A "two-hit" hypothesis has been proposed, involving the accumulation of fat in the liver ("first hit"), together with a "second hit" that produces oxidative stress. Hepatic steatosis has been recognised as the first of two hits in the pathogenesis of NASH, since the presence of oxidizable fat within the liver is enough to trigger lipid peroxidation[13]. However, many patients with fatty liver do not progress to steatohepatitis. Potential second hits for the evolution towards NASH include all mechanisms contributing to the development of inflammation and fibrosis. The presumed factors initiating second hits are oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines (principally tumour necrosis factor alpha), and hormones derived from adipose tissue (adipocytokines)[12]. The progression of liver disease in CLDs presents with a common histopathological pathway which is the formation and accumulation of fibrosis leading to the development of progressive distortion of the hepatic architecture that is the hallmark of evolution to cirrhosis. Liver fibrosis is the result of chronic injury and it appears to play a direct role in the pathogenesis of hepatocellular dysfunction and portal hypertension[14,15]. Development of fibrosis is a progressive process starting from minimal fibrosis limited to the portal tracts, followed by more extensive fibrosis with septa expanding into the liver parenchyma, which can form bridges between two portal tracts or portal tracts and central veins, eventually ending in complete cirrhotic nodules. In patients with CLDs precise definition of the hepatic fibrosis stage is of paramount importance to evaluate the prognosis and the follow-up of the hepatic disease and to decide the need for antiviral therapy in HBV and HCV chronic infections. In CLDs liver biopsy has always been the gold standard for evaluating presence, type and stage of liver fibrosis and to characterize necroinflammation. This procedure, however, presents some limitations since it is invasive, costly and difficult to standardize. Recently, there has been increasing interest in non-invasive assessment of liver fibrosis by using surrogate markers measurable in the peripheral blood or by using instrumental devices, but some concerns about their large-scale clinical use have been raised, based on their performance and validation. This article aims to review the current status of the literature regarding non-invasive assessment of liver fibrosis in CLDs, considering its limitations and advantages. Finally, decisional algorithms to be applied to the most validated and reliable methods in clinical practice are here proposed.

Table 1 METAVIR and Ishak staging systems for liver fibrosis

Description	METAVIR (F)	Ishak (S)
No fibrosis	0	0
Portal fibrosis without septa	1	1-2
Portal fibrosis with few septa	2	3
Septal fibrosis without cirrhosis	3	4
Cirrhosis	4	5-6

Portal fibrosis is a stellate enlargement of portal tracts without any bridging fibrosis on the biopsy sample. Few septa means at least one fibrous septum on the core biopsy. Theoretically, a fibrous septum is a bridge of connective tissue between two portal tracts, a portal tract and a centrolobular vein, or between two centrolobular veins. Septal fibrosis means that the liver biopsy is crossed by several septa; the transition between F2 and F3 by METAVIR or S3 and S4 by Ishak begins when there are more fibrous septa than portal tracts without septa on the biopsy. Cirrhosis means that liver tissue is mutilated by nodular fibrosis that delineates hepatocytes nodules.

HISTOLOGICAL SYSTEMS TO STAGE LIVER FIBROSIS

Several semiquantitative scoring systems have been proposed to stage fibrosis and to grade necroinflammation in the liver. The Ishak's system is a revised version of the older histological activity index[16,17]. It describes grading and staging as two separate items and liver fibrosis is classified as absent (0), mild (1-2), moderate (3-4) and severe/cirrhosis (5-6). This classification system is mainly applied to hepatitis B and C. The METAVIR scoring system for staging has been frequently used in recent times particularly for chronic hepatitis C (Table 1)[18]. Brunt classification of fibrosis assessment is generally used for NASH and it includes five stages: stage 0, no fibrosis; stage 1, zone 3 perisinusoidal or pericellular fibrosis, focally or extensively present; stage 2, zone 3 perisinusoidal or pericellular fibrosis with focal or extensive periportal fibrosis; stage 3, zone 3 perisinusoidal or pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis; stage 4, cirrhosis[19]. All these scoring systems have some limits, being semiquantitative, not linear and prone to intra- and inter-observer variation and to sampling variability.

LIVER BIOPSY: IS IT A GOLD OR A SILVER STANDARD?

Liver biopsy has long been the gold standard for staging of

Table 2 Pros and Cons of liver biopsy in staging of hepatic fibrosis

PROS	CONS
Staging of liver fibrosis	Invasiveness (pain, bleeding)
Grade of necroinflammation	Cost (hospitalization)
Steatosis (common in hepatitis C)	Sampling errors
Iron overload (common in hepatitis C)	Possibly refused by patient, concern of physician
Comorbidities (autoimmunity stigmates)	Static data, no information on fibrogenesis

liver fibrosis in CLDs. Liver biopsy has the advantage of obtaining direct information not only about fibrosis, but also about many useful parameters, such as inflammation, necrosis, steatosis, iron or copper deposits. Furthermore, it allows the identification of suspected or unexpected cofactors and comorbidities. However, biopsy is associated with potential morbidity and mortality and has several limitations (Table 2). A single liver biopsy provides static data but with no information on fibrogenesis and fibrolysis that characterise the dynamic processes related to extracellular matrix (ECM) metabolism. Moreover, many recent studies clearly indicate that liver biopsy is prone to sampling errors and may underestimate the amount of liver fibrosis. Cirrhosis could be missed on a single blind percutaneous liver biopsy in 10%-30% of cases[20,21]. When three different liver samples were analyzed, the percentage of correct diagnoses increased from 80% to 100%[22]. In more recent times, Regev *et al*[23] have shown that samples obtained from the right and left lobes of the liver during laparoscopy give different fibrosis staging in one third of cases, with a concordance rate of more than 90% between two experienced pathologists. Other studies have analyzed agreement/disagreement among pathologists. Although the use of more standardized scoring systems, such as those of the Ishak's, METAVIR's and Brunt's classifications, has improved the inter-observer and intra-observer variability, there are still several factors that may significantly influence the reliability of a liver biopsy. The size of the liver sample is very important, especially if we consider that a hepatic sample of 15 mm length represents 1/50000 of the whole parenchyma. Colloredo *et al*[24] have carefully analyzed the impact of the sample size on a correct staging of liver fibrosis in patients with hepatitis C. By reducing progressively the dimensions of the same liver biopsy, they reported that the smaller was the sample analyzed, the milder was the diagnosis made by the pathologist in relation to the stage of fibrosis. Other studies have reported that the type and the size of needle used are also important. The Tru-Cut needle was found to be superior to the Menghini needle, particularly for the diagnosis of more advanced fibrosis[25]. The use of a thicker needle ameliorates the accuracy of the diagnosis but also implies an increased risk of bleeding and perforation for the patient. Interestingly, Rousselet *et al*[26] reported that the degree of experience of the pathologist, as indicated by longer duration of practice or belonging to an academic setting, may have an outstanding impact on the diagnostic interpretation of liver biopsy, even higher than that determined by the one related to sample size. Another shortcoming of liver biopsy is its cost. A cost-benefit analysis showed that in the US the cost of a liver biopsy is 1032 USD and it could rise to 2745 USD when complications occur[27].

Table 3 Features of an adequate liver biopsy sample

Length (mm)	Portal tracts (n°)	Ref.
15	5	[28,29]
20	11	[30]
25	NA	[31]
Bigger is better	NA	[32]

NA: Not available.

LIVER BIOPSY: CONSENSUS AMONG PATHOLOGISTS?

Pathologists have tried to define the features (including length and number of complete portal tracts) of an adequate liver biopsy sample able to reduce the risk of misclassification of liver fibrosis (Table 3). Some authors would suggest that an adequate liver biopsy sample should contain more than 5 portal tracts and be at least 15 mm in length[28,29]. Other studies reported a higher threshold for optimized accuracy. Guido and Rugge have produced a critical review of the literature concerning the use of liver biopsy in chronic viral hepatitis[30]. They suggest that liver biopsy is very often flawed by unacceptable methodological limits and that a biopsy sample of 20 mm or more containing at least 11 complete portal tracts should be considered reliable for adequate grading and staging. Other authors have recommended even bigger samples, up to 25 mm in length[31]. Scheuer has recently concluded that "bigger is better"[32].

LIVER BIOPSY: CONSENSUS AMONG CLINICIANS?

The pathologist's need for obtaining a liver sample of adequate size is in contrast with the patient's need for a procedure causing limited pain and risks. Liver biopsy may in fact be a risky procedure for some patients, particularly for those with more advanced liver fibrosis. Indeed, one third of patients experience pain at the time of the procedure, and the proportion of 0.3%-0.6% of cases presents with serious adverse events like bleeding and even death in decompensated cirrhosis[33]. A French survey which interviewed 1177 general practitioners concluded that liver biopsy may be refused by up to 59% of patients with hepatitis C and that 22% of the physicians share the same concern for the invasiveness of the procedure[34]. On this topic, a survey assessing the consensus among Italian hepatologists on when and how to take a liver biopsy in chronic hepatitis C showed great divergence in the management of the same subgroup of patients[35]. A nationwide survey about assessment of liver fibrosis in hepatitis C among French hepatologist showed

that liver biopsy was still systematically performed by only 4% of respondents. Guidelines for the clinical use of non-invasive methods for assessment of liver fibrosis were required by 95% of the respondents[36].

THE IDEAL NON-INVASIVE METHOD FOR LIVER FIBROSIS

In view of all the shortcomings regarding liver biopsy, in the last decade clinical investigators have been searching for non-invasive methods for accurate information about liver fibrogenesis activity and fibrosis stage in patients with CLDs. Fibrosis is a structural change in the liver that accompanies chronic injury; fibrogenesis refers to the production of ECM. Fibrogenesis increases in response to injury and is essential to tissue repair. The key step in the pathophysiology of liver fibrogenesis is the balance between ECM deposition and removal. An excess of ECM produced after injury stimulates fibrolysis which is mediated by several specific matrix metalloproteinases (MMPs). The hepatic stellate cells (HSCs) are the major source of ECM[14]. Guidelines and Recommendations indicate that staging of liver fibrosis is the most important parameter for the definition of prognosis and for the subsequent management of the patient with CLD[37,38]. Natural history studies indicate that, if only an insignificant rate of patients without fibrosis will develop cirrhosis in the following 5 years, this percentage goes up to 20% for cases with portal fibrosis and to more than 40% for cases with septal fibrosis[39]. Moreover, the decision whether to start an antiviral therapy in cases of chronic viral hepatitis is highly influenced by the staging of liver fibrosis, since treatments are usually long, costly and cause side effects. Identification of patients with cirrhosis is essential to start screening for end-stage liver complications, including esophageal varices (OV) and HCC. International guidelines have defined two stages of liver fibrosis that significantly modify the management of the patients in clinical practice[37,38]: (1) Significant fibrosis, defined as a liver fibrosis stage (F) $\geqslant$ 2 according to METAVIR for hepatitis C or (S) $\geqslant$ 2 according to Ishak for hepatitis B. Significant fibrosis is a definitive indication to start antiviral therapy in chronic hepatitis B and in chronic hepatitis C due to difficult-to-treat genotypes (HCV-1 and HCV-4). For patients infected with HCV genotype 2 or 3 histological definition is not necessary except for those cases with relative contraindications, not motivated or elderly age. The recent Italian Guidelines on the management of chronic hepatitis B have underlined the importance of the stage of liver fibrosis not only in deciding who to treat, but also in deciding the first choice treatment: interferon for mild-moderate fibrosis and nucleoside/nucleotide analogues for cirrhosis, especially if decompensated[38]. (2) Hepatic cirrhosis, defined as liver fibrosis stage of (F) 4 by METAVIR and of (S) 6 by Ishak. Cirrhosis, even when fully compensated and still clinically occult, requires a different and more specific management than simple chronic hepatitis, including screening for OV with annual gastroscopy and for HCC with ultrasound and alpha-fetoprotein every 6 mo.

Table 4 Features of the ideal non-invasive method for liver fibrosis

Reliable (high diagnostic accuracy)
Widely available (simple, least expensive)
Providing information on both fibrosis stage and fibrogenesis activity
Validated by large-scale studies
Validated by independent studies (different authors from the proposing study)
Validated in various etiologies of CLDs (HCV, HBV, ALD, NAFLD)
Identifying clinically important fibrosis stages (significant fibrosis and cirrhosis)

CLDs: Chronic liver diseases; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

The ideal marker test would be able to accurately stage disease and also be sensitive to changes in fibrosis induced by the natural course of disease progression or by therapy (Table 4). Non-invasive methods for detecting liver fibrosis may be divided in two main groups: markers measured in peripheral blood, which could be single parameters or panels combining more parameters, and a technical device that measures the liver stiffness through transient elastography (fibroscan).

SERUM NON-INVASIVE MARKERS OF LIVER FIBROSIS

Among the proposed markers in the literature, some are directly linked to the modifications in ECM turnover occurring during fibrogenesis, the so-called "direct markers", while others reflect alterations in hepatic function but do not directly reflect ECM metabolism, the so-called "indirect markers"[14,15]. The direct markers of liver fibrosis include several glycoproteins (hyaluronan, laminin, human cartilage glycoprotein 39), the collagens family (procollagen Ⅲ, type Ⅳ collagen), the collagenases and their inhibitors and a number of cytokines connected with the fibrogenetic process (TGF-β1, TNF-α). These markers have a pathophysiologic rationale since they may be an expression of either deposition or removal of ECM, thus giving information on its metabolism. They may potentially be used not only to stage liver fibrosis, but also to assess the speed of liver fibrogenesis with the most relevant prognostic value, and also to estimate and monitor the efficacy of and the response to antifibrotic drugs. A limitation to the clinical use of direct markers of liver fibrosis is that they are not routinely available in all hospital settings. The indirect markers of liver fibrosis are biochemical parameters that are measurable in the peripheral blood. They are an indirect expression of liver damage and have a statistical association with liver fibrosis stage. While direct markers of liver fibrosis reflect the process of fibrogenesis, indirect markers satisfy the request for a simple and easy-to-perform marker. Both direct and indirect markers for liver fibrosis may be single or a combination of parameters (Tables 5 and 6). Most of them have been proposed and validated in chronic hepatitis C. Table 7 describes the accuracy of various

Table 5 Single serum non-invasive markers for liver fibrosis

Direct markers	Indirect markers
Hyaluronic acid	Platelet count
Laminin	AST, ALT
Procollagen Ⅲ	γGT
Type Ⅳ Collagen	γ-globulins
Metalloproteinases	Albumin
Inhibitors of metalloproteinases	Prothrombin time

serum non-invasive markers for liver fibrosis as reported in the literature. The performance of non-invasive markers is usually expressed as sensitivity, specificity, positive and negative predictive values (PPV, NPV), accuracy, and compared area under the receiving operating characteristic curve (AUROC).

Hyaluronic acid has been extensively studied in hepatitis C while few studies are available in other etiologies. Overall, a rather good accuracy of this marker in the different CLDs has been reported for detection of significant fibrosis, with an AUROC ranging from a minimum of 0.82 to a very good 0.92[40-46]. In a study conducted in 326 patients, the AUROC was 0.86 and the specificity was 95% for significant fibrosis while the AUROC was 0.92 and the specificity was 89.4% for cirrhosis when a cut off level of 110 μg/L was used[45]. However, another cohort study with more than 400 cases has reported an AUROC of only 0.73 for significant fibrosis[42]. In the same study, cirrhosis could be excluded with excellent NPV and sensitivity (100%) and with excellent AUROC (0.97) using a cut off level of 50 μg/L. Similar results were reported in another study of 486 patients in which hyaluronic acid levels < 60 μg/L excluded cirrhosis with 99% NPV[40]. In ALD the performance of hyaluronic acid for significant fibrosis varied significantly[43,46] while the marker showed very good performance for cirrhosis, with an AUROC of 0.93[46]. The results of a study conducted in 79 patients with NAFLD were also encouraging, since hyaluronic acid had a 0.92 AUROC value for cirrhosis[44]. On the basis of its good accuracy, especially for exclusion of cirrhosis, hyaluronic acid has also been used in panels combining other serum non-invasive markers for liver fibrosis. Recently it has been proposed in combination with AST-to-platelet ratio index (APRI) in hepatitis B. In this study, a combination of APRI > 1.5 and of hyaluronic acid > 300 ng/mL had 98.9% specificity and 93.7% PPV[47]. Laminin is another component of ECM that has been studied as a non-invasive marker. Serum levels of laminin have been used by several authors as a non-invasive parameter to assess liver fibrosis in ALD patients as well as in those presenting with viral hepatitis and hemochromatosis[48]. This determination, however, was progressively discontinued as it did not demonstrate superiority to those of other components of the ECM such as hyaluronic acid. It showed 77% accuracy for detection of significant fibrosis in hepatitis C in a detailed study on 243 patients with CLD[49]. With regard to NAFLD, however, the use of laminin serum levels could be further investigated since a single report, which investigated liver fibrosis in 30 overweight patients, showed a rather good accuracy (87%)[50]. Among the collagens, type Ⅳ collagen has been investigated as surrogate marker of liver fibrosis. Type Ⅳ collagen has been studied in hepatitis C and a good performance for significant fibrosis has been reported (AUC = 0.83)[51]. Murawaki *et al*[52] have compared the diagnostic performance of type Ⅳ collagen with that of hyaluronic acid in hepatitis C and reported the superiority of the latter marker. The role of type Ⅳ collagen has also been investigated in 112 patients with NAFLD and its performance has been compared with hyaluronic acid[53]. The results showed a better diagnostic accuracy for type Ⅳ collagen (0.828 *vs* 0.797 AUROC, respectively). Metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) have also been proposed as surrogate markers of liver fibrosis. Those reported to have some clinical impact include MMP-2 and TIMP-1[54]. Boeker *et al*[54] reported a very high performance of MMP-2 in detecting cirrhosis (0.97 AUROC). Unfortunately, it has been difficult to obtain good standardization of the method for routine clinical use. Some authors proposed panels of direct non-invasive markers with the aim of increasing the accuracy of the single parameters. Fibrometer combines age, platelets, prothrombin index, AST, α-2-macroglobulin, hyaluronan and urea. In a few studies, the AUROC for significant fibrosis has been reported as 0.89 in hepatitis C, raising to an excellent 0.943 in patients with NAFLD[55,56]. Patel *et al*[57] proposed fibrospect which combines hyaluronic acid, TIMP-1 and α-2-macroglobulin. It showed an AUC 0.832 for METAVIR stages F2-F4 fibrosis with PPV and NPV of 74.3% and 75.8%, respectively. Another model, named Hepascore, combines bilirubin, γGT, hyaluronan, α-2-macroglobulin, age, and sex, and showed in hepatitis C and ALD a quite good performance for diagnosis of significant fibrosis, ranging from 0.78 to 0.85, and excellent performance for cirrhosis, ranging from 0.89 to 0.92[58,59]. Unfortunately, for both these combination panels large-scale, independent validation studies are lacking. The European Liver Fibrosis (ELF) study group proposed a panel of markers combining age, hyaluronan, type Ⅲ collagen and TIMP-1. In a cohort study of more than one thousand patients with a variety of CLDs the panel detected moderate or advanced fibrosis (Scheuer stages 3, 4) with a 0.77 to 0.94 AUROC in hepatitis C and ALD, respectively[60]. The panel has also been recently validated in 196 patients with NAFLD, with 0.90 AUROC for detection of severe fibrosis, that could increase to 0.98 when the original panel was combined with simple markers[61]. Similar results in terms of accuracy have been recently obtained in 112 consecutive pediatric patients with NAFLD[62].

AST-to-ALT ratio (AAR) was one of the first non-invasive markers proposed. It is easily available and without any cost but it showed a highly variable performance in the studies conducted on HCV patients: sensitivity was between 31.5% and 81.3%, specificity was between 55.3% and 97% and accuracy ranged from 60%-83.6%[63,64]. Another concern about this test may be that it does not identify significant fibrosis but only cirrhosis. In a prospective study, we have also validated AAR in 110 patients with chronic hepatitis B and we obtained 78.9% accuracy for the diagnosis of cirrhosis[65]. AST-to-platelet ratio in-

Table 6 Combinations of serum parameters for non-invasive diagnosis of liver fibrosis

Marker	Description	Settings in which validation exists	Ref.
AST/ALT	AST to ALT ratio	HCV, HBV	[63-65,71,72]
APRI	AST to platelets ratio index	HCV, HBV, HIV/HCV	[64-67,72,75-80]
Forns' index	Age, BMI, γGT, cholesterol	HCV, HBV, HIV/HCV	[67,69,72,75]
Fibrotest	Age, gender, α-2-macroglobulin, γGT, haptoglobin, apolipoprotein A1, total bilirubin	HCV, HBV, ALD, NAFLD, HIV/HCV	[65,67,72,76-80]
ELF	Age, hyaluronic acid, type Ⅲ procollagen, TIMP1	HCV, ALD, NAFLD	[60-62]
Hepascore	Bilirubin, γGT, hyaluronic acid, α-2-macroglobulin, age, sex	HCV	[58,59]
Lok index	AST, ALT, platelets, INR	HCV	[68]
Fibroindex	AST, platelets, g-globulins	HCV	[71,72]
Fibrometer	Age, AST, platelets, hyaluronan, INR, α-2-macroglobulin, urea	HCV	[55,56]
Fibrospect	α-2-macroglobulin, hyaluronan, TIMP1	HCV	[57]
Fib-4	Age, AST, ALT, platelets	HCV, HBV, HIV/HCV	[73-75]

APRI: AST-to platelet ratio index; ELF: European liver fibrosis study group.

Table 7 Performance of several serum non-invasive markers for liver fibrosis (single or combination) as expressed as AUROC

Serum marker	Significant fibrosis	Cirrhosis	Ref.
Hyaluronic acid	0.73-0.92	0.85-0.97	[40-47]
Laminin	0.82	NA	[48,49]
Type Ⅳ collagen	0.83	NA	[51-53]
MMP-2	0.59	0.97	[54]
TIMP-1	0.71	0.90	[54]
ELF	0.77-0.94	NA	[60-62]
AAR	NA	0.51-0.83	[63-65,71,72]
Forns' index	0.75-0.86	NA	[67,68,70,72]
APRI	0.69-0.88	0.61-0.94	[15,64-67,72,76-80]
Fibrotest	0.74-0.87	0.71-0.87	[15,65,67,72,76-80]
Fibroindex	0.74-0.83	NA	[71,72]
Fibrometer	0.89-0.96	NA	[55,56]
Fibrospect	0.83	NA	[57]
Fib-4	0.79-0.85	0.80-0.91	[73-75]
Hepascore	0.82-0.85	0.90-0.94	[58,59]

AUROC: Area under the receiving operating characteristic curve; MMP-2: Metalloproteinase 2; TIMP-1: Tissue inhibitor of metalloproteinases 1; ELF: European liver fibrosis study group; AAR: AST-to-ALT ratio; APRI: AST-to-platelet ratio index.

dex (APRI) is a simple and cheap ratio between AST and platelets, easily available in the clinical practice. It classifies both significant fibrosis and cirrhosis but around 50% of the cases result as unclassified. APRI performance is variable among the studies on hepatitis C: sensitivity ranges between 41% and 91%, specificity between 47% and 95% and accuracy between 60% and 82.7% for significant fibrosis; for cirrhosis, sensitivity ranges between 38.4% and 65.8%, specificity between 86.7% and 93% and accuracy between 60% and 88.4%[15,66,67]. We have also validated APRI in hepatitis B, obtaining 76.1% accuracy for the diagnosis of significant fibrosis and 79.2% for the diagnosis of cirrhosis[65]. Most recently, APRI has been modified into Lok index by adding alanine aminotrasferase (ALT) and international normalized ratio (INR), with further improvement of the diagnostic accuracy, particularly for cirrhosis[68].

Forns' index is a simple panel resulting from the combination of age, γGT, cholesterol and platelets. It does not give any information about cirrhosis, but only about significant fibrosis. Around half of the cases cannot be classified. In hepatitis C, the accuracy reported in various studies was variable (between 50% and 85%)[67,69]. We have also validated Forns' index in hepatitis B, obtaining 64.8% accuracy for the diagnosis of significant fibrosis[65]. It has been suggested that Forns' index might be less accurate in patients with HCV genotype 3 which is associated with very low cholesterol levels[70]. However, this has not been confirmed by other data[67]. In a study performed on 3690 patients with chronic hepatitis C, a combination panel derived from platelets, AST, and γ-globulin named Fibroindex showed 0.83 AUROC in predicting significant fibrosis[71]. However, following validation studies it showed a lower performance[72]. Another combination of simple markers named Fib-4 was recently proposed and it uses platelets, ALT, AST and age. It showed good performance for detection of severe fibrosis (0.85 AUROC) and even better for the diagnosis of cirrhosis (0.91 AUROC) in chronic hepatitis C[73]. The performance of the panel was also evaluated in a cohort of patients with chronic hepatitis B, with similar accuracy for diagnosis of significant fibrosis (0.81 AUROC)[74]. The validity of Fib-4 as a non-invasive marker for liver fibrosis has also been investigated in patients with HCV/HIV coinfection and the reported accuracy was 0.79 for significant fibrosis and 0.80 for cirrhosis[75]. Fibrotest is a patented test that combines γGT, total bilirubin, haptoglobin, α-2-macroglobulin, apolipoprotein A1, age and gender[76]. To date, it is the most validated non-invasive method for liver fibrosis in various etiologies: HCV, HBV, ALD, NAFLD and HIV/HCV coinfected. Between 2001 and 2008 more than 60 scientific studies have investigated fibrotest and 20 of them are independent with respect to the group that have commercialized the test. Overall, independent studies have investigated fibrotest in more than 3000 patients with CLD, mostly hepatitis C. The accuracy reported ranges from 70%-85%[15,67,76]. Fibrotest has been applied to hepatitis B patients and the accuracy reported varies between 83.3% and 87.3% for significant fibrosis and between 86.1% and 94.4% for the diagnosis of cirrhosis[65,77]. In HIV/HCV coinfected patients AUROC was 0.85 for significant fibrosis and 0.87 for cirrhosis[78]. Fibrotest was also validated in ALD, with excellent results, especially for cirrhosis (0.84

AUROC for significant fibrosis and 0.95 AUROC for cirrhosis)[79]. Fibrotest was also applied in 170 patients with NAFLD and the AUROC for significant fibrosis was 0.86[80]. These results in HIV/HCV coinfected, ALD and NAFLD cases need, however, further confirmation from independent groups. Some conditions may alter the result of fibrotest, including Gilbert syndrome and hemolysis. In these cases the clinician should be cautious in the interpretation of the result and the test should be repeated. Overall, among the various serum markers proposed in the literature, APRI and fibrotest are the most validated in all etiologies, and also validated in many independent studies.

TRANSIENT ELASTOGRAPHY (FIBROSCAN)

Apart from serum markers, another method for non-invasive assessment of liver fibrosis is the measurement of liver stiffness[81]. Transient elastography is measured through a device that is called fibroscan (Echosens, Paris) which is composed of an ultrasound transducer probe mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency are transmitted by the transducer, inducing an elastic shear wave that propagates through the underlying tissues. Pulse-echo ultrasound acquisition is used to follow the propagation of the shear wave and to measure its velocity, which is directly related to tissue stiffness: the stiffer the tissue, the faster the shear wave propagates. Transient elastography measures liver stiffness in a volume that is approximately a cylinder 1 cm wide and 4 cm long, between 2.5 cm and 6.5 cm below the skin surface. This volume is at least 100 times bigger than a biopsy sample. Fibroscan examination is painless, rapid (less than 5 min) and easy to perform at the bedside or in the outpatient clinic. The examination is performed on a non-fasting patient lying flat on his/her back, with the right arm tucked behind the head. The probe transducer is placed on the skin, between the rib bones at the level of the right lobe of the liver where biopsy would be performed. The operator performs 10 valid acquisitions and then the software of fibroscan calculates the median value. The software itself determines whether each measurement is successful or not. Results are expressed in kilo-Pascals (kPa). Liver stiffness values range from 2.5-75 kPa. The results are immediately available and are operator-independent[82]. The exam can be done after a short learning curve (about 100 examinations). The validity of a fibroscan result should be based on two important parameters: (1) the interquartile range (IQR), which reflects the variability of the validated measures, and should not exceed 30% of the median value; (2) the success rate, that is the percentage of valid measurement, should be at least 60%. Despite the exam being relatively easy to perform, the clinical interpretation of results should always be in the hands of an expert clinician who should have at his disposal all clinical information regarding the patient. The result of the fibroscan is given according to cut-off values expressed in kPa: according to the various studies, presence of significant fibrosis is defined by a cutoff value of 7.1 to 8.7, and cirrhosis is diagnosed by a cutoff value of 12.5 to 14.5[83,84]. In various studies, the accuracy of fibroscan results were similar to that of serum non-invasive markers for the diagnosis of significant fibrosis, sometimes with inadequate figures (< 80%). On the other hand, fibroscan showed excellent performance for the diagnosis of cirrhosis (Table 8)[85]. Liver stiffness measurements can be difficult in obese patients or in those with narrow intercostal space and impossible in patients with ascites[81]. Failure rates range between 2.4% and 9.4% in the different studies[81-83,86]. Factors associated with inter- and intra-observator variability were BMI > 25, high grade hepatic steatosis and mild fibrosis (F0-F1 by METAVIR)[82]. A single report suggested that transaminase flares during chronic HBV infection may alter the result of fibroscan because of high flogosis and recruitment of inflammatory cells into the liver parenchyma[87]. Interestingly, a report suggested that acute viral hepatitis increases liver stiffness measured by fibroscan, thus the authors recommend that the extent of necroinflammatory activity needs to be carefully considered in future studies, particularly in patients with absent or low-stage liver fibrosis[90]. Non-invasive assessment of liver fibrosis with fibroscan has also been applied to ALD with 0.91 AUROC for significant fibrosis and 0.92 for cirrhosis[91]. Table 9 summarizes the main limitations of fibroscan. A recent meta-analysis concluded that for the diagnosis of significant fibrosis, transient elastography cannot be used sufficiently in clinical practice. Inclusion of transient elastography in an algorithm with a combination of non-invasive serum markers may be considered[92]. Transient elastography can be used in clinical practice as an excellent tool for the confirmation of cirrhosis when other clinical signs and examinations are non-decisive.

Table 8 Accuracy of fibroscan for the diagnosis of significant fibrosis and cirrhosis

Ref.	Etiology	Accuracy for ⩾ F2	Accuracy for F4
[81]	HCV	88	99
[83]	HCV	83	95
[84]	HCV	79	95
[86]	HCV	80	96
[87]	HCV	NA	95
[88]	HBV	87	88
[89]	HBV	90	94

COMBINATION ALGORITHMS AND IMPLEMENTATION OF NON-INVASIVE METHODS FOR LIVER FIBROSIS IN CLINICAL PRACTICE

The accuracy of most non-invasive methods for liver fibrosis showed variability among different studies and is still considered inadequate to substitute for liver biopsy and for implementation of non-invasive markers for liver fibrosis in clinical practice[15,29,93]. Some preliminary

Table 9 Limitations of fibroscan in clinical practice

Difficult to perform in obese patients (5% rate failure)
Inter-observer and intra-observer variability influenced by liver steatosis
Influence of ALT flares (HBV reactivation)
Lower performance for diagnosis of significant fibrosis

studies suggested that accuracy of non-invasive methods may improve when they are combined in diagnostic algorithms. We have recently proposed an approach that combines APRI and fibrotest sequentially with the aim of increasing the diagnostic accuracy[67]. This is a rational approach for the use of non-invasive markers for liver fibrosis in clinical practice. Indeed, these markers are used when they present with adequate accuracy, while liver biopsy is used only in those patients in which non-invasive markers showed inadequate accuracy. This approach has been named SAFE (Sequential Algorithms for Fibrosis Evaluation) biopsy and its aim is to reduce the number of liver biopsies that are necessary to correctly stage liver fibrosis and to minimize misclassified cases. Through stepwise modeling, two algorithms were developed with the aim of correctly classifying the two stages of liver fibrosis that are clinically significant: (1) significant fibrosis, (2) cirrhosis. The modeling of the algorithms was aimed at achieving > 90% accuracy and minimizing misclassified cases. In the model APRI has been used as first line test since it is cheap and simple, fibrotest has been used as second line test since it is costly and more complex. Liver biopsy has been used only as third line test in those cases in which the two non-invasive markers did not show adequate accuracy and/or in unclassified cases (only for APRI) (Figures 1 and 2). The modeling of the stepwise algorithms was based on the predictive values of the single markers. In the algorithm for significant fibrosis (Figure 1), 0.5 cut-off of APRI had low NPV to exclude significant fibrosis, while 1.5 cut-off showed high PPV to diagnose significant fibrosis. Similarly, 0.49 cut-off of fibrotest showed high PPV to diagnose significant fibrosis, whereas values less than 0.48 could not accurately exclude significant fibrosis. In the algorithm for cirrhosis (Figure 2), 1 cut-off for APRI showed high NPV to exclude cirrhosis, while 2 cut-off did not show sufficient PPV to diagnose cirrhosis. Similarly, 0.48 and 0.75 cut-offs of fibrotest showed good NPV and PPV, respectively, for cirrhosis, while intermediate values could not give accurate diagnosis.

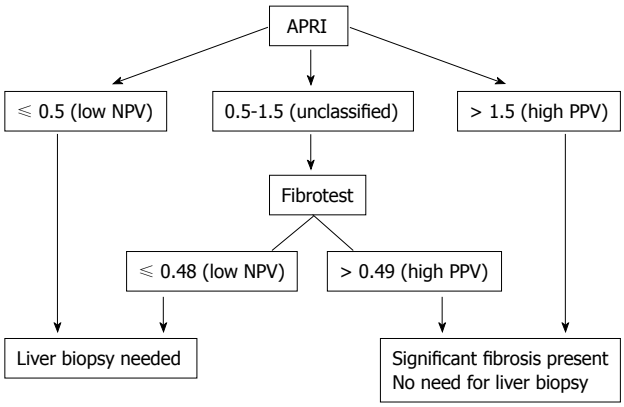

Figure 1 The SAFE-biopsy algorithm for significant fibrosis (≥ F2 by METAVIR). The figure reports the cut-offs used for APRI and Fibrotest in the decisional tree.

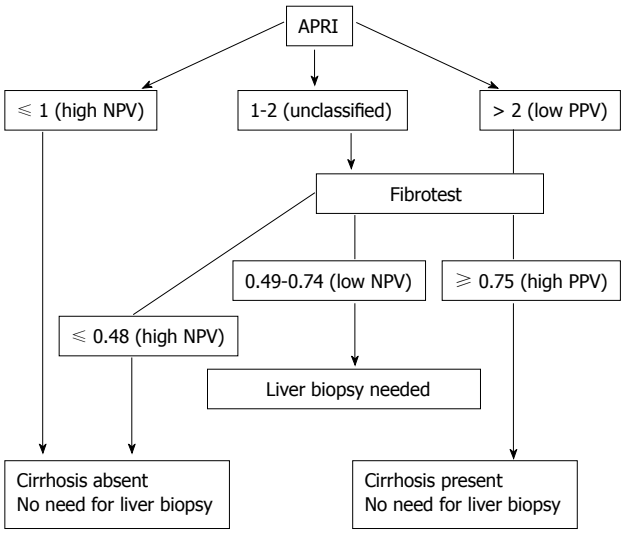

Figure 2 The SAFE-biopsy algorithm for cirrhosis (F4 by METAVIR). The figure reports the cut-offs used for APRI and Fibrotest in the decisional tree.

IMPLEMENTATION OF SAFE BIOPSY IN CLINICAL PRACTICE

In clinical practice, SAFE biopsy can provide the following responses: (1) Presence of significant fibrosis, then indication to administer antiviral therapy; (2) Presence of liver cirrhosis, then indication to specific follow-up with abdominal ultrasound, α-fetoprotein and gastroscopy; (3) absence of cirrhosis; (4) liver biopsy needed to correctly stage hepatic fibrosis.

The main concept of SAFE biopsy is that liver biopsy cannot be completely avoided but can be markedly reduced and limited to those cases in which serum markers for liver fibrosis do not show enough accuracy. Indeed, SAFE biopsy may avoid the diagnostic funnel represented by liver biopsy and it may stimulate general practitioners and patients to perform the initial screening for CLD. With this approach, liver biopsy and non-invasive markers for liver fibrosis are not antagonists, but they are agonists towards the common goal of correctly classifying liver fibrosis. SAFE biopsy has been recently validated in a multicentre, international study on serum non-invasive markers for liver fibrosis. This study, named SAFE protocol protocol, has enrolled more than 2500 cases of patients with CLD in whom APRI and fibrotest were available and liver histology was used as reference standard. The centers involved were from Italy, US, France and Romania. To date, this is the largest independent study on non-invasive methods for liver fibrosis. We have recently presented the results on 2035 cases with hepatitis C and they have confirmed high accuracy and high number of saved liver biopsies[94] (Table 10). The results of an interim analysis conducted on 210 HBV patients also showed high ac-

Table 10 Main features of SAFE biopsy[67,94] for significant fibrosis and cirrhosis in 2035 HCV cases

	Significant fibrosis	Cirrhosis
Sensitivity (%)	100	92.7
Specificity (%)	77	90.4
Accuracy (%)	90	93
AUROC	0.9	0.92
Saved biopsies (%)	47	82

SAFE: Sequential algorithms for fibrosis evaluation; AUROC: Area under the receiving operating characteristic curve.

curacy (> 90%) of SAFE biopsy algorithms for both significant fibrosis and cirrhosis, with a percentage of saved liver biopsies ranging from 45%-82%. We have also compared in 1013 HCV cases the performance of SAFE biopsy with another two algorithms combining non-invasive markers for liver fibrosis that were then proposed: Fibropaca algorithm, based on concordance of Forns' index, APRI and fibrotest; Leroy algorithm, based on concordance of APRI and fibrotest[95-97] (Table 11). Fibropaca algorithm and SAFE biopsy showed a similar accuracy but the latter saved more liver biopsies and allowed us to perform a minor number of non-invasive markers, with a consequent saving in terms of costs. The main advantages of SAFE biopsy include a larger first level screening of liver fibrosis, higher patient compliance and lower screening costs. In some specific settings, SAFE biopsy may show even more efficient results when compared with the diagnostic funnel represented by liver biopsy alone.

ALGORITHMS FOR IMPLEMENTATION IN CLINICAL PRACTICE

Castera *et al*[83] have recently proposed an algorithm which combines fibrotest and fibroscan with the aim of increasing the accuracy of the single non-invasive methods in hepatitis C. This algorithm results in an increased accuracy, especially for the diagnosis of significant fibrosis. A recent collaborative study was aimed at comparing the algorithm combining fibroscan and fibrotest (named Bordeaux algorithm) and SAFE biopsy in 302 patients with hepatitis C[98] (Table 12). The results showed that the Bordeaux algorithm saved more liver biopsies for diagnosis of significant fibrosis, although both algorithms saved a similar number of overall liver biopsies, and Bordeaux algorithm showed a higher overall accuracy for diagnosis of cirrhosis. On the other hand, Bordeaux algorithm uses fibrotest and fibroscan in all patients, while SAFE biopsy uses fibrotest in a subgroup of patients that are not well classified by APRI, which has virtually no cost. The two algorithms could be used for large scale screening of liver fibrosis and the choice of the algorithm may be based on the local availability of the non-invasive methods. Interestingly, the use of either fibroscan or fibrotest has been recently recommended in France by the Haute Autorité de Santé for the first line assessment of liver fibrosis in patients with hepatitis C without comorbidities[85]. Figure 3A and

Table 11 Comparison of the performance of SAFE biopsy[67,94], Fibropaca algorithm[96] and Leroy algorithm[97]. Results are expressed as percentages

	SAFE biopsy for diagnosis of		Fibropaca algorithm for diagnosis of		Leroy algorithm for diagnosis of
	⩾ F2	F4	⩾ F2	F4	⩾ F2
APRI needed	100	100	100	100	100
Forns needed	0	0	100	0	0
Fibrotest needed	41.7	57.6	100	100	100
Sensitivity	100	81.8	85.5	72.7	89.6
Specificity	78.2	92.4	89.9	96.7	97.8
Accuracy	90	91.2	87.6	94	93.5
Saved biopsies	43.8	79.1	51.7	76.2	29.2

⩾ F2: Significant fibrosis; F4: Cirrhosis; APRI: AST-to-platelet ratio index.

Table 12 Comparison of the performance of Bordeaux algorithm[98] and SAFE biopsy[67,94]. Values are expressed as percentages

	Bordeaux algorithm		SAFE biopsy	
	⩾ F2	F4	⩾ F2	F4
APRI needed	0	0	100	100
Fibrotest needed	100	100	43.7	61.9
Fibroscan needed	100	100	0	0
Accuracy	91	93	94	87
Biopsies saved	71.9	78.8	48.3	74.8

B show a rational proposal for the use of non-invasive methods for liver fibrosis in clinical practice, based on the local availability of the different methods and on their performances. A combination approach for clinical use has also been proposed by others[99]. Non-invasive methods for liver fibrosis and combination algorithms may be of paramount importance for the monitoring of progression of liver disease. Indeed, if it is acceptable to perform a liver biopsy at time 0, it is inconceivable however to perform a liver biopsy every year to monitor liver fibrosis progression, while this is feasible with non-invasive methods for liver fibrosis. According to local availability of the methods and attainment of non-invasive markers by the clinician, two different approaches may be used: (1) to fix the value with combined use of biopsy and non-invasive markers at time 0 and then monitoring with non-invasive markers; (2) to use non-invasive markers and then perform a liver biopsy when clinically necessary (Figure 4A and B).

MONITORING OF EFFICACY OF ANTIVIRAL THERAPIES

Apart from the diagnosis of liver fibrosis stage, few recent studies have focused on the possible use of non-invasive methods for liver fibrosis in the monitoring of antiviral therapies. Indeed, especially in hepatitis B, antiviral therapies may be long-term, such as treatments with nucleoside/nucleotide analogues, and the clinician may want to know not only the biochemical or virological response, but also and more appropriately the histological

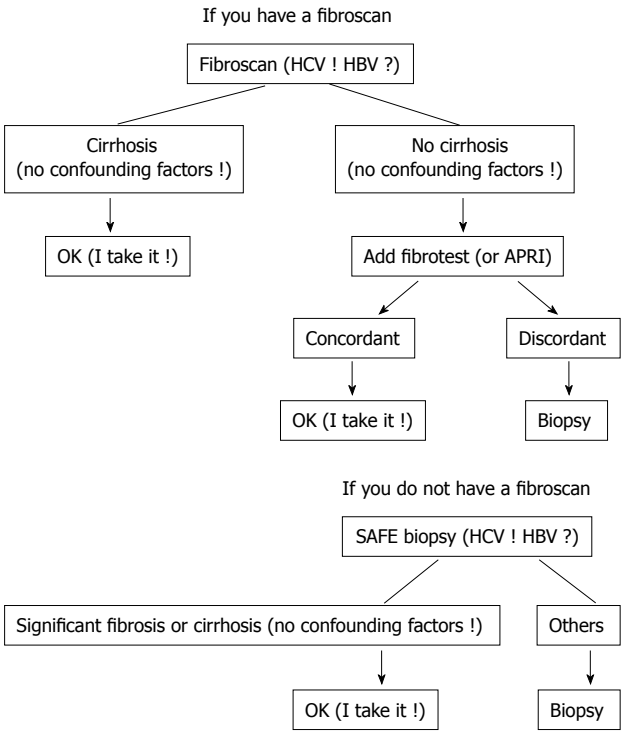

Figure 3 Diagnostic algorithms for implementation of non-invasive methods for liver fibrosis in clinical practice based on the local availability of the most validated methods.

response. Initial reports have shown that both fibrotest and fibroscan values change significantly during and after antiviral therapy in both hepatitis C and B[100-102]. Indeed, a significant improvement in fibrotest and fibroscan value has been reported in patients who achieve sustained virological response (SVR) *vs* those without SVR, and in some cases this was also maintained for 12 mo after therapy[101]. This may mean that there is a regression of liver fibrosis with antiviral treatment but further prospective, large-scale studies are needed.

MONITORING OF LIVER DISEASE COMPLICATIONS

A very attractive application of non-invasive methods for liver fibrosis may be the monitoring of liver disease complications to predict clinical events in compensated cirrhosis. Preliminary results suggest that liver stiffness values in cirrhotic patients may increase as liver disease is more advanced. In a retrospective study of 711 patients with CLD (95 with histologically-proven cirrhosis), liver stiffness values significantly correlated not only with the Child-Pugh score but also with clinical parameters (past history of bleeding varices or ascites, HCC), biochemical parameters (platelets, INR, factor V, albumin and bilirubin) and others (2-3 grade OV, splenomegaly on sonography, nodular surface, heterogeneous parenchyma) of liver disease severity[86]. Cut-off values of 27.5, 37.5, 49.1, 53.7 and 62.7 kPa had > 90% NPV for the presence of grade 2-3 OV, Child-Pugh scores B or C, past history of ascites, HCC and esophageal bleeding,

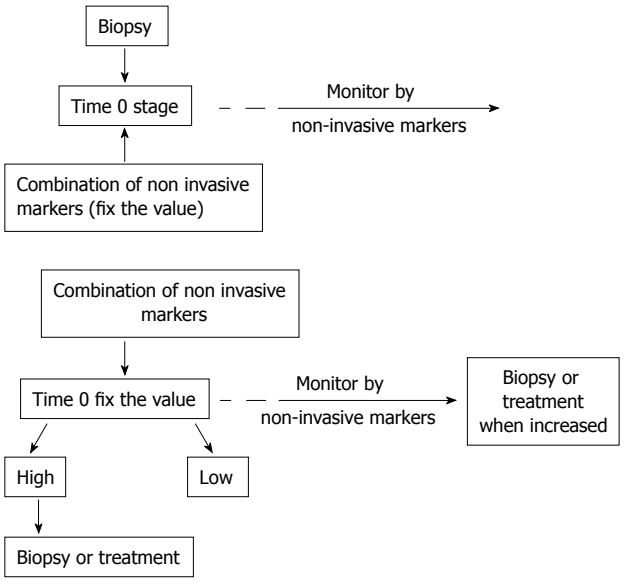

Figure 4 Perspectives for the use of non-invasive markers for long term monitoring of chronic liver diseases.

respectively. More recently, Vizzuti *et al*[103] reported a rather high sensitivity (90%) of fibroscan for prediction of OV with 17.6 kPa cut-off. Other preliminary studies have suggested that some non-invasive markers for liver fibrosis could predict the presence of OV. Sanyal *et al*[104] reported high NPV for excluding grade 2-3 varices when platelets were > 150 000/mm^3. Giannini *et al*[105] reported a good sensitivity (91.5%) with an overall accuracy of 86% for diagnosis of OV with a 909 cut-off of platelet count to spleen diameter ratio. A recent multicenter, international study was aimed at investigating in 510 consecutive cirrhotic patients the role of 7 simple non-invasive markers for liver fibrosis in predicting the presence of OV of any grade and of grade 2-3 OV[106]. The markers analyzed were platelets, AAR, Lok index, APRI, Forns' index, Fib-4, Fibroindex. Presence of grade 2-3 OV could be excluded with > 96% NPV by a specific cut-off of Lok index (1.5). None of the tests were able to predict the presence of grade 2-3 OV due to low PPV. A combination of Lok index (cutoff 0.9) and Forns' index (8.5) could predict the presence of OV of any grade with 88% PPV, 83% accuracy and 0.82 AUC. The conclusion was that, even if simple non-invasive markers for liver fibrosis cannot be a substitute for endoscopy for OV screening, they may be used to stratify cirrhotics by risk. In a recent prospective study of 298 patients with chronic hepatitis C, the performance of fibroscan, fibrotest and simple serum markers for detection of cirrhosis and its complications have been assessed[107]. The authors concluded that fibroscan is the most accurate method for diagnosis of cirrhosis but it cannot replace endoscopy for screening of OV. These preliminary findings are promising but need to be confirmed in long-term prospective follow-up studies.

Several recent studies have reported a correlation between liver stiffness values and portal hypertension,

assessed by measurement of hepatic venous pressure gradient (HVPG) which is considered the gold standard for the diagnosis and staging of portal hypertension[103,108-110]. Carrion *et al*[108] reported a close direct correlation between liver stiffness values and HVPG in 124 HCV-infected liver transplant recipients. More recently, Vizzutti *et al*[103] reported similar results in 61 patients with HCV-related severe CLD (METAVIR F3-F4). Other authors have failed to find similar results[111].

THE FUTURE: GENETICS FOR THE IDENTIFICATION OF PATIENTS WITH CHRONIC LIVER DISEASES AT RISK OF PROGRESSION

The identification of patients at high risk of developing progressive liver disease on the basis of genetic profile may be extremely useful in the future. A recent collaborative study used seven genetic variants to identify patients with hepatitis C at risk for developing cirrhosis, based on the analysis of paired liver biopsies. A cirrhosis risk score (CRS) was calculated on the basis of seven single nucleotide polymorphisms and the patient's gender[112]. In this case, increasing CRS was associated with fibrosis progression in HCV patients presenting with no liver fibrosis. CRS genetic signature could potentially be a useful prognostic indicator of those patients with HCV infection most likely to develop fibrosis progression and/or cirrhosis.

HIGHLIGHTS

Staging of liver fibrosis is essential in clinical practice for the management of patients with CLDs. Nowadays liver biopsy can no longer be considered the exclusive tool for the diagnosis of liver fibrosis since the available data support a rational use of the most validated non-invasive methods for liver fibrosis and especially of their combination algorithms. This is particularly true for chronic hepatitis C, where an adequate validation of some non-invasive methods for liver fibrosis exists. Non-invasive methods for liver fibrosis, when combined, may reduce by 50%-80% the number of liver biopsies needed for correctly classifying hepatic fibrosis. However, liver biopsy cannot be completely avoided but should be used in those cases in which non-invasive methods show poor accuracy. In clinical practice, the choice of the non-invasive method and, especially, of the combination algorithms may depend on their performance and on local availability. Further studies, especially in chronic hepatitis B, ALD and NAFLD, are needed to better assess performance of non-invasive markers in these settings and to develop rational algorithms for implementing non-invasive assessment of liver fibrosis. Future studies should also focus on non-invasive monitoring of antiviral treatment efficacy and cirrhosis complications, and genetic studies for precocious identification of patients who are at high risk of developing end-stage liver diseases.

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REVIEW

Review of salt consumption and stomach cancer risk: Epidemiological and biological evidence

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Abstract

Stomach cancer is still the fourth most common cancer; thus, it remains an important public health burden worldwide, especially in developing countries. The remarkable geographic variations in the rates of stomach cancer indicate that dietary factors, including a range of food groups to which salt and/or nitrates have been added, may affect stomach cancer risk. In this paper, we review the results from ecologic, case-control and cohort studies on the relationship between salt or salted foods and stomach cancer risk. The majority of ecological studies indicated that the average salt intake in each population was closely correlated with gastric cancer mortality. Most case-control studies showed similar results, indicating a moderate to high increase in risk for the highest level of salt or salted food consumption. The overall results from cohort studies are not totally consistent, but are suggestive of a moderate direct association. Since salt intake has been correlated with *Helicobacter pylori* (*H pylori*) infection, it is possible that these two factors may synergize to promote the development of stomach cancer. Additionally, salt may also cause stomach cancer through directly damaging gastric mucus, improving temporary epithelial proliferation and the incidence of endogenous mutations, and inducing hypergastrinemia that leads to eventual parietal cell loss and progression to gastric cancer. Based on the considerable evidence from ecological, case-control and cohort studies worldwide and the mechanistic plausibility, limitation on salt and salted food consumption is a practical strategy for preventing gastric cancer.

Key words: Disease prevention; *Helicobacter pylori* infection; Salt consumption; Stomach cancer

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Wang XQ, Terry PD, Yan H. Review of salt consumption and stomach cancer risk: Epidemiological and biological evidence. *World J Gastroenterol* 2009; 15(18): 2204-2213 Available from: URL: http://www.wjgnet.com/1007-9327/15/2204.asp DOI: http://dx.doi.org/10.3748/wjg.15.2204

INTRODUCTION

Stomach cancer is the fourth most common cancer and is the third leading cause of cancer death worldwide[1-3]. The estimated number of stomach cancer cases worldwide was 933 900 in 2002, with two-thirds occurring in developing countries[3]. Tremendous variation in both incidence and mortality rates exists across geographic regions, with > 10-fold differences observed between low-risk and high-risk areas[4]. Although stomach cancer incidence rates have been decreasing slowly over recent decades in China, it was estimated that there were 0.4 million new cases diagnosed and 0.3 million deaths from this malignancy in 2005[5]. Therefore, this disease remains an important public health burden throughout the world, especially in developing counties including China.

Several risk factors for stomach cancer have been identified, including *Helicobacter pylori* (*H pylori*) infection, salt-preserved foods, dietary nitrite, smoking, alcohol, obesity, radiation, and family history[6,7]. Researchers also found that the incidence rates of stomach cancer varied across different geographic regions and this variation may be associated with genetic, lifestyle or environmental

factors, including diet[8]. Salt intake was first reported as a possible risk factor for stomach cancer in 1959[9]. In some early studies, using refrigerators for food storage, which may be an indicator of less salted food consumption or decreased salt intake, was found to be correlated with a reduction in stomach cancer rates[10,11], leading researchers to hypothesize that salt intake may play a role in the development of stomach cancer. A Japanese ecological study suggested a nearly linear correlation between the cumulative mortality rate of stomach cancer and the median 24 h urine salt excretion level[12,13]. Experimental studies[14,15], including rodent models, have also suggested that salt may play an important role in the etiology of stomach cancer. Based on the available experimental and epidemiological data, a report from World Health Organization (WHO)/Food and Agriculture Organization (FAO) Expert Consultation in 2003 concluded that "salt-preserved foods and salt probably increase the risk of stomach cancer"[16].

The purpose of this paper was to review the current literature on salt consumption and the risk of stomach cancer. We obtained the relevant papers and identified our literature search through PubMed from SCI papers. All cohort papers were selected with cohort size more than 2000; case-control papers were filtered out with case sample size around or more than 100. At the end, we summarized the evidence from epidemiological perspectives regarding salt intake and stomach cancer risk.

EPIDEMIOLOGICAL STUDIES OF SALT AND STOMACH CANCER RISK

When evaluating epidemiologic studies on the relationship between salt or salted food consumption and stomach cancer risk, it is essential to consider the diversity of salted foods. Some studies analyzed overall dietary salt intake, whereas others evaluated stomach cancer risk associated with salt intake in various categories, such as table salt or salted fish.

Ecologic studies

Several ecologic studies reported positive associations between different indicators of salt consumption and stomach cancer mortality at the population level[17-21]. In an ecologic study of 24 countries, median urinary sodium levels, ascertained on randomly selected samples from each country, were significantly correlated with stomach cancer mortality (r = 0.70 in men; 0.74 in women) (both $P < 0.001$)[17]. Another study evaluated correlations between both salt intake and 24 h urinary sodium excretion and stomach cancer mortality among men in four geographic regions of Japan, and reported a strong correlation between stomach cancer mortality and salt excretion, but not with dietary salt intake[21]. An ecologic study of stomach cancer mortality in 65 Chinese counties observed significant, yet modest, correlations for intake of salt-preserved vegetables (r = 0.26 in men and 0.36 in women)[18]. A Japanese study administered a 38-item food frequency questionnaire to a sample of 634 men and the wives of 373 of these men from five districts in Japan. The rank correlation coefficient between gastric cancer mortality and pickled vegetable consumption was 0.36[19]. In a similar study of 207 Japanese men and the wives of 165 of the men, average daily sodium consumption, estimated using a 3 d weighed food record, was correlated with stomach cancer mortality rates (partial rank r = 0.45)[20].

In summary, the majority of ecological studies indicated that the average salt intake in each population was closely correlated with gastric cancer mortality. However, employing dietary assessment methods in these studies has some limitations, such as variation of the questionnaire validity in different population, and use of the same composition table calculating salt intake in diverse dietary cultures. One validated questionnaire used by one population may not be appropriate or valid for another population; thus in population based studies, dietary estimates may not be highly accurate and the association between dietary factors and disease may be much compromised. Applying the same composition table to calculate the same food in different dietary culture may cause bias, since the same food in different areas may have different salt content. For example, the regional average salt content of miso in 39 regions of 20 prefectures across Japan ranged from 9.1% to 18.2%[22]. Furthermore, as with all ecologic studies, diet and stomach cancer were neither measured nor analyzed on the individual level. Rather, the diets of sampled individuals were used to represent entire populations or geographic regions. Thus, misclassification is an obvious concern. Furthermore, associations observed at the population level cannot be assumed to hold at the individual level, and causality cannot be inferred from this type of study.

Case-control studies

Forty two case-control studies on salt consumption in relation to stomach cancer are presented in Table 1[23-64]. Of these, twenty two studied overall salt, table salt or sodium[23-44], twelve estimated salted/dried fish and/or salted fish gut or cod roe[25,39,45-54], six investigated salted or pickled vegetables[25,32,52-55], three focused on salty snacks[41,56,57], and twelve studied salted foods in general[29,30,32,52,54,58-64].

Among the sixteen studies that estimated overall dietary salt or sodium intake, eight in Puerto Rico, Spain, Korea, Italy, Mexico, China (two) and USA have shown strong statistically significant increases in risk (OR = 1.5-5.0 for the highest intake levels)[28-31,33,34,36,43]. Seven of them reported statistically non-significant OR of 1.1 to 1.5 for consumption in the upper half of overall salt or sodium intake[24-27,32,35,44], and the remaining study reported no association[23]. Six of the studies specifically examined the use of table salt, with three studies (in Belgium, England, and Poland) reporting statistically significant increases in risk with OR = 1.6, 1.8, 6.2, respectively[38,40,42]. Two other studies each reported statistically non-significant odds ratios of 1.5 for consumption of the upper half of the table salt intake distribution[37,39]; the remaining study reported no

Table 1 Summary of 45 case-control studies that evaluated the association between salt consumption and stomach cancer risk

Author and year	No. of cases	No. of controls	Factors evaluated	Exposure levels and OR (95% CI)[1]
SALT AND SODIUM INTAKE				
Modan *et al*, 1974: Israel[23]	166	429	Salt	No association
Risch *et al*, 1985: Canada[24]	246	246	Salt	No association
You *et al*, 1988: China[25]	564	1131	Salt (per capita household)	≤ 13 kg/yr, ≤ 19 kg/yr, ≤ 20 kg/yr, > 20 kg/yr 1.0, 1.2, 1.1, 1.1 (0.8-1.4); NS
Negri *et al*, 1990: Italy[26]	526	1223	Salt	Low, intermediate, salty 1.0, 1.3, 1.2 (0.8-1.7); NS
Wu-Willians *et al*, 1990: USA[27]	137 male	137	Add salt	Rarely, often, always 1.0, 1.4, 1.2 (Na); NS
Nazario *et al*, 1993: Puerto Rico[28]	136	151	Salt	≤ 6.979 g/wk; 6.98-18.66 g/wk; 18.67-43.26 g/wk; ≥ 43.27 g/wk 1.0, 2.9, 4.5, 5.0 (2.1-12.0); $P < 0.05$
Ramón *et al*, 1993: Spain[29]	117	234	Salt	Quartiles 1 through 4 1.0, 1.2, 1.8, 2.1 (1.2-7.1); P for trend < 0.01
Lee *et al*, 1995: Korea[30]	213	213	Salt	Tertile 3 *vs* 1 3.7 (1.1-12.5); $P < 0.05$
La Vecchia c *et al*, 1997: Italy[31]	746	2053	Salt	Low, intermediate or high 1.0, 1.5 (1.0-2.2); $P < 0.05$
Ye *et al*, 1998: China[32]	272	544	Salt	0.25 kg/m; > 0.25 kg/m 1.0, 1.3 (1.0-1.6); NS
López-Carrillo *et al*, 1999: Mexico[33]	220	752	Salt	Never, sometimes (adding salt after tasting the food) Positive association; $P < 0.05$
Liu *et al*, 2001: China[34]	189	189	Heavy salt	Low to high half 1.0, 2.0 (1.3-3.2); $P < 0.05$
Machida-Montani *et al*, 2004: Japan[35]	122	235	Salt	Tertiles 1 through 3 1.0, 1.3, 1.5 (0.6-3.7); NS
Qiu *et al*, 2004: China[36]	103	133	Salt	Positive association; $P < 0.05$
La Vecchia *et al*, 1987: Italy[37]	206	474	Table salt	Tertile 3 *vs* 1 1.5 (Na); NS
Tuyns *et al*, 1988: Belgium[38]	293	2851	Table salt	Never, sometimes, always 1.0, 1.0, 1.8 (1.2-2.8); $P < 0.05$
Buiatti *et al*, 1989: Italy[39]	1016	1159	Table salt	Seldom, always 1.5 (1.3-1.9); NS
Coggon *et al*, 1989: England[40]	95	190	Table salt	Low to high half 1.0, 6.2 (2.0-18.9); $P < 0.05$
Boeing *et al*, 1991: Germany[41]	143	579	Table salt	No association
Boeing *et al*, 1991: Poland[42]	741	741	Table salt	Low to high half 1.0, 1.6 (1.2-2.3); $P < 0.05$
Graham *et al*, 1990: USA[43]			Sodium intake	≤ 73.2 g/mo; 73.2-98.8 g/mo; ≤ 98.9-127.3 g/mo; > 127.3 g/mo
	186 male	181		1.0, 1.8, 2.6, 3.1 (1.7-5.8); P for trend = 0.001 ≤ 66.9; 67.0-88.5; > 88.5
	107 female	104		1.0, 1.8, 4.7 (2.3-9.6); P for trend = 0.0001
Harrison *et al*, 1997: USA[44]		132	Sodium intake	Low to high half
	60 intestinal			1.0, 1.3 (0.8-1.9); NS
	31 diffuse			1.0, 1.4 (0.9-2.1); NS
SALTY FOODS				
Salted fish				
Haenszel *et al*, 1972: Japan[45]	220	440	Salted/dried fish	None, use both, 2 times/mo; 3-5 times/mo; 6 times/mo 1.0, 2.0, 1.5, 2.5, 2.6 (Na); $P < 0.05$
Haenszel *et al*, 1976: Japan[46]	783	1566	Salted/dried fish	None; < 4 times/mo; 4-9 times/mo; ≥ 10 times/mo 1.0, 1.1, 1.1, 1.2 (Na); NS
Tajima *et al*, 1985: Japan[47]	93	186	Salted/dried fish	Low to high half 1.0, 2.6 (Na); $P < 0.01$
You *et al*, 1988: China[25]	564	1131	Sated fish	≤ 0.5 kg/yr, ≤ 1 kg/yr, > 1 kg/yr 1.0, 1.0, 1.4 (0.8-1.5); NS
Buiatti *et al*, 1989: Italy[39]	1016	1159	Salted/dried fish	Tertile 3 *vs* 1 1.4 (Na); P for trend = 0.001
Kato *et al*, 1990: Japan[48]				< 2-3 times/wk; ≥ 2-3 times/wk
	289 male	1247	Salted/dried fish	1.0, 1.2 (0.9-1.7); NS
			Salted fish gut, cod roe	1.0, 1.5 (1.1-2.1); $P < 0.05$
	138 female	1767	Salted/dried fish	1.0, 0.7 (0.5-1.0); NS
			Salted fish gut, cod roe	1.0, 0.5 (0.3-1.0); NS
González *et al*, 1991: Spain[49]	354	354	Salted fish	Low to high half 1.0, 1.5 (0.9-2.6); NS
Palli *et al*, 1992: Italy[50]		1159	Salted/dried fish	Tertile 3 *vs* 1

	68 cardia 855 others			1.7 (0.9-3.1); NS 1.5 (1.2-1.8); NS
Hansson *et al*, 1993: Sweden[51]	338	669	Salted fish	None, ⩽ 0.9 times/mo; ⩽ 3 times/mo; ⩽ 7 times/mo; ⩽ 11 times/mo 1.0, 1.0, 0.9, 0.9, 1.3 (0.8-2.1); NS (adolescence) None, ⩽ 0.9 times/mo; ⩽ 3 times/mo; ⩽ 7 times/mo 1.0, 1.0, 0.8, 0.8 (0.5-1.3); NS (20 yr prior to interview)
Kim *et al*, 2002: Korea[52]	136	136	Salted fish	Tertiles 1 through 3 1.0, 0.8, 0.8 (0.4-1.6); NS
Cai *et al*, 2003: China[53]	381	222	Salty fish	< times/mo, < 3 times/wk, ⩾ 3 times/wk 1.0, 1.0, 5.5 (1.4-19.5); NS
Strumylaite *et al*, 2006: Lithuania[54]	379	1137	Salted fish	Almost do not use, 1-3 times/mo 1.0, 0.7 (0.5-0.9); *P* for trend = 0.002
Salted vegetable				
You *et al*, 1988: China[25]	564	1131	Sated vegetables	< daily, daily 1.0, 1.1 (0.7-1.8); NS
Ye *et al*, 1998: China[32]	272	544	Salted vegetables	< 2 kg/yr; > 2 kg/yr 1.0, 1.4 (1.1-1.8); *P* < 0.05
Kim *et al*, 2002: Korea[52]	136	136	Salted vegetables	Tertiles 1 through 3 1.0, 0.9, 1.5 (0.8-2.9); NS
Xibin *et al*, 2002: China[55]	210	630		Low to high half
			Pickled or salted vegetables	1.0, 4.0 (1.6-9.8); *P* < 0.05
			Preference for a high salt vegetables	1.0, 2.6 (1.6-4.3); *P* < 0.05
Cai *et al*, 2003: China[53]	381	222	Pickled vegetables	< times/M, < 3 times/w, ⩾ 3 times/wk 1.0, 1.3, 1.8 (1.0-3.0); *P* for trend = 0.038
Strumylaite *et al*, 2006: Lithuania[54]	379	1137	Pickled vegetables with salt and oil	Almost do not use, 1-3 times/mo, ⩾ 1-2 times/wk
			Pickled vegetables with salt and vinegar	1.0, 0.6, 0.8 (0.6-2.1); NS
			Salted mushrooms	Almost do not use, 1-3 times/mo, ⩾ 1-2 times/wk 1.0,0.7, 0.8 (0.6-1.0); NS 1-3 times/mo, ⩾ 1-2 times/wk 1.0, 1.6 (1.1-2.4); NS
Salted snacks				
Boeing *et al*, 1991: Germany[41]	143	579	Pretzels, salty snacks	Tertiles 1 through 3 1.0, 0.7, 1.5 (1.0-2.2); NS
Ward *et al*, 1999: Mexico[56]	220	752	Salty snacks	Never, ⩽ 2, > 2 times/mo 1.0, 1.3, 1.8 (1.2-2.8); *P* for trend = 0.008
Chen *et al*, 2002: Nebraska[57]	124	449	Salty snacks	Quartiles 1 through 4 1.0, 1.4, 1.2, 0.7 (0.3-1.6);NS
Salted foods in general				
Hu *et al*, 1988: China[58]	241	241	Salted and fermented soya paste	< 2 kg/yr; > 2 kg/yr 1.0, 1.5 (1.0-2.2); NS
Kono *et al*, 1988: Japan[59]	139	2852	Salty foods	None or 1-3 times/mo; 1-3 times/mo; once/do more 1.0, 0.8, 1.4 (Na): NS
Demirer *et al*, 1990: Turkey[60]	100	100	Salted foods	Less than once or twice/wk; once or twice/wk *vs* 1.0, 3.8 (2.1-6.9); *P* < 0.001
Hoshiyama *et al*,1992: Japan[61]	294	294 (general population) 202 (hospital control)	Salty foods	No, moderate, yes 1.0, 1.7, 2.3 (1.5-3.4); *P* < 0.01 1.0, 1.3, 1.1 (0.7-1.9); NS
Ramón *et al*, 1993: Spain[29]	117	234	Pickled foods	Quartiles 1 through 4 1.0, 1.2, 2.1, 3.7 (Na); *P* for trend < 0.01
Ji *et al*, 1998: China[62]	1124	1451	Salted foods	Occasionally, sometimes, frequently 1.0, 1.4, 1.7 (1.3-2.4); *P* for trend = 0.001
Lee *et al*, 1995: Korea[30]	213	213	Salted side dishes	Tertile 3 *vs* 1 4.5 (2.5-8.0); *P* < 0.05
Ye *et al*, 1998: China[32]	272	544	Salted fermented sea foods	< 1.5 kg/yr; > 1.5 kg/yr 1.0, 1.6 (1.2-2.0); *P* < 0.01
Kim *et al*, 2002: Korea[52]	136	136	Salty foods	Tertiles 1 through 3 1.0, 1.1, 0.9 (0.4-1.8); NS
De Stefani *et al*, 2004: Uruguay[63]	240	960	Salted meat	Tertiles 1 through 3 1.0, 1.3, 2.0 (1.4-2.9); *P* for trend = 0.0003
Campos *et al*, 2006: Colombia[64]	368	431	Salting meals before tasting	No, yes 1.0, 3.5 (1.6-7.3); *P* for trend = 0.001
Strumylaite *et al*, 2006: Lithuania[54]	379	1137	Salted meat	Almost do not use, 1-3 times/mo, ⩾ 1-2 times/wk 1.0, 1.5, 3.0 (2.2-4.0); *P* for trend < 0.001

[1]OR, Odds ratio; CI: Confidence interval; Na: No association.

Table 2 Summary of 11 cohort studies that evaluated the association between salt consumption and stomach cancer risk

Author and yr	Size of cohort	No. of cases	Length of follow-up (yr)	Factors evaluated	Exposure levels and RR (95% CI)[1]
Salt					
Nomura *et al*, 1990: USA[65]	7990 male	150	4	Table salt/shoyu	Never-seldom, after tasting, always 1.0, 1.4, 1.0 (0.6-1.6); NS
van den Brandt *et al*, 2003: Netherlands[66]	120852	282	6.3	Dietary salt	Quintiles 1 through 5 1.0, 1.5, 1.0, 1.5, 1.2 (0.8-1.8); NS
				Table salt	Never, seldom, sometimes, often/very often 1.0, 1.1, 0.7, 0.9 (0.6-1.4); NS
Tsugane *et al*, 2004: Japan[67]			12	Salt	Quintiles 1 through 5
	18684	358			Male: 1.0, 1.7, 2.0, 2.3, 2.2 (1.5-3.4); *P* for trend < 0.001
	20381	128			Female:1.0, 0.9, 1.0, 0.6, 1.3 (0.8-2.3); NS
Shikata *et al*, 2006: Japan[68]	2476	93	14	Dietary salt	< 10.0, 10.0-12.9, 13.0-15.9, ≤ 16.0 1.0, 2.1, 1.9, 2.7 (1.4-5.2); *P* for trend = 0.01
Sjödahl *et al*, 2008: Sweden[69]	73133	313	18	Dietary salt	Low to high half 1.0, 1.0 (0.7-1.4); NS
Salty foods					
Kneller *et al*, 1991: USA[70]	17633 male	75	20	Salted fish	Never, < 1, ≥ 1 1.0, 1.0, 1.9 (1.0-3.6); NS
Galanis *et al*, 1998: USA[71]	11907	108	14.8 (average)	Dried or salted fish	None, 1 or more times/wk
				High-salt foods	1.0, 1.0 (0.6-1.7); NS None, 1-3 times/wk, 4 or more times/wk 1.0, 1.0, 1.1, (0.7-1.8); NS
Ngoan *et al*, 2002: Japan[72]	13000	116	10	Salted food	Low, median, high 1.0, 1.0, 1.4 (0.6-3.2); NS
Kim *et al*, 2004: Japan[73]		400	10	Salted food	Quartiles 1 through 4
	20300			(traditional type)	Male:1.0, 2.0, 2.5, 2.9 (1.8-4.7); *P* for trend < 0.0001
	21812				Female: 1.0, 1.7, 1.3, 2.4 (1.3-4.4); *P* for trend = 0.007
Tokui *et al*, 2005: Japan[74]	110792		12	Preference for salty food	No, a little, somewhat, much, very much
		574			Male: 1.0, 0.9, 1.1, 1.1, 1.4 (0.7-2.8); NS
		285			Female: 1.0, 1.6, 1.8, 1.5, 1.9 (0.6-5.8); NS
				Dried or salty fish	None, 1-2/mo, 1-2/wk, 3-4/wk, 1+/d
		574			Male: 1.0, 0.9, 0.9, 0.9, 1.1 (0.7-1.8); NS
		285			Female:1.0, 0.6, 0.7, 0.7, 0.9 (0.5-1.6); NS
Kurosawa *et al*, 2006: Japan[75]	8035	76	11	Salted food	Low, intermediate, high 1.0, 4.0, 5.4 (1.8-16.3); *P* for trend < 0.01

[1]RR: Relative risk.

association[41].

Of the twelve studies that estimated salted fish intake, four found strong statistically significant increases in risk (OR = 1.4-5.5 for the highest intake levels)[39,45,47,48]. One Japanese study reported a statistically significant increase in risk for high consumption of salted fish gut and cod roe in males, but not females, and no significant association for salted/dried fish for both genders[48]. Seven other studies reported statistically non-significant correlations[25,46,49-53]; the remaining study reported a statistically significant inverse association with odds ratios of 0.7 for consumption in the upper half of the salted fish intake distribution[54].

Six studies in Table 1 examined salted vegetables; of these, three reported statistically significant increases in risk with higher intakes of salted vegetables[32,53,55], the remaining three studies in China, Korea and Lithuania showed no relationship to stomach cancer risk[25,52,54]. Additionally, three studies reported on salted snacks. Of them, only one study in Mexico reported a statistically significant relationship to stomach cancer[56], with the other two reporting no substantial associations[41,57].

Twelve studies examined consumption of salted soya paste, salted side dishes and salty foods in general[29,30,32,52,54,58-64]. Nine of them observed a moderate increase in risk with higher consumption (OR = 1.6-4.5)[29,30,32,54,60-64], the remaining three reported no association[52,58,59].

In summary, many case-control studies found similar results, indicating a moderate to high increase in risk for the highest level of salt or salted food consumption. Given the large number of studies that reported data on salt, sodium and salty foods consumption, some inconsistent results were to be expected. The inconsistencies may be due, at least in part, to the retrospective assessment of salt exposure, which might have changed after the diagnosis of stomach cancer. Furthermore, the degree to which each of these measures reflects total salt intake varies, and it is therefore not surprising that results would vary.

Cohort studies

Eleven cohort studies, investigating salt or salted food consumption and stomach cancer risk in the US, Japan, Sweden, and the Netherlands have produced inconsistent results (Table 2)[65-75]. When viewed separately in the Table, the results were inconsistent for both salt intake and intake of salty foods. Four Japanese studies reported

statistically significant associations (range of RR = 2.2-5.4 for the highest intake level)[67,68,73,75], including one study that reported significantly elevated risks in both men and women after 10 years follow up of 20 300 men and 21 812 women[73]. Another study, conducted in rural Japan with 8035 subjects and 76 stomach cancer deaths, reported a significantly elevated relative risk for the most frequent intake of highly salted foods compared with the least frequent intake (RR = 5.4; 1.8-16.3; *P* for trend < 0.01)[75]. In a study that examined 18 684 men and 20 381 women and included 486 histologically confirmed stomach cancer cases (358 men and 128 women), there was a dose-dependent association between salt consumption and stomach cancer risk in men (*P* for trend < 0.001), but not in women (*P* for trend = 0.48)[67]. Shikata *et al*[68] categorized 2476 subjects into four groups according to daily salt intake. After 14 years of follow-up, the age- and sex-adjusted incidence was significantly higher in the second to fourth groups than in the first group (RR = 2.4, 95% CI: 1.2-4.7; RR = 2.1, 95% CI: 1.0-4.3; RR = 3.0, 95% CI: 1.5-5.8, respectively). With the exception of these positive findings, the remaining seven cohort studies showed no substantial associations[65,66,69-72,74]. It is perhaps noteworthy that four studies with statistically significant positive results were conducted in Japan, which may be related to a potentially higher range of salt intake in that country.

In summary, some cohort studies suggest that a higher intake of salt or of salted food, as estimated by validated food frequency questionnaires, may be directly associated (or at least indirectly linked) with subsequent development of stomach cancer. Although the overall results from cohort studies are not totally consistent, they are suggestive of a moderate direct association.

EVIDENCE ON INTERACTIONS BETWEEN SALT OR SALTED FOODS AND HELICOBACTER PYLORI INFECTION

Even though *H pylori* infection is the strongest risk factor for stomach cancer, it cannot completely explain the worldwide distribution of this disease. It is very important to evaluate the potential joint effects of *H pylori* infection and other factors, including salt intake, in stomach cancer carcinogenesis.

Few epidemiological studies have investigated *H pylori* infection in relation to salt consumption. In an international ecologic study[76], statistically significant correlations between national *H pylori* infection rates and national salt excretion levels were found in older (age 50-64) men and women (r = 0.73 and r = 0.83, respectively) and in younger (age 20-34) men (r = 0.73), but not in younger women (r = 0.52). A cross-sectional study of 634 Japanese men[77] reported that daily consumption of miso soup was associated with the prevalence of *H pylori* (OR = 1.60, *P*< 0.05). Similarly, increasing consumption of pickled vegetables was associated with increased *H pylori* infection risk (OR = 1.90 for the highest level, *P* for trend = 0.02). Despite limitations inherent in these types of studies, they can nevertheless provide information on potential associations between salted foods and *H pylori* infection in humans, which may be evaluated more fully in case-control and cohort studies.

Three previous epidemiological studies have examined the potential synergistic relationship between salt consumption and *H pylori* infection in the development of stomach cancer; however, the results are inconsistent[35,68,78]. A case-control study in Japan analyzing the independent and joint effects of diet and *H pylori* infection found that subjects with *H pylori* infection and with high salt intake (OR = 14.2) had a higher odds ratio compared with subjects with *H pylori* infection and low salt intake (OR = 9.7) (reference group was no *H pylori* infection and low salt intake), but there was not a statistically significant interaction between the two risk factors[35]. A Korean case-control study investigating the role of salt and *H pylori* infection in stomach cancer found that subjects with *H pylori* infection and high salt consumption had a 10-fold risk of early stomach cancer compared with subjects without *H pylori* infection and with a low salt consumption (*P* = 0.047)[78]. These two case-control studies have some limitations, including issues of possible recall bias and misclassification. For example, both *H pylori* and salt intake were assessed after the development of stomach cancer. Advancing stomach cancer can combine with the loss of infection characterized by a fall in circulating anti-*H pylori* antibodies and changes in salt exposure (or in recollection of dietary exposures prior to the onset of disease). Only one cohort study, conducted in Japan, evaluated the potential interaction between diet and *H pylori,* and found that the positive association between increased salt intake and gastric cancer was statistically significant among subjects with *H pylori* infection only[68]. The relative risks were similar, however, and the authors note that findings for dietary salt were most pronounced in subjects who had both *H pylori* infection and atrophic gastritis. The three studies discussed above have relatively small sample sizes, ranging from 69 to 122 stomach cancer cases, and thus the estimation of relative risk is imprecise and results should be interpreted cautiously, especially in the analyses of effect modification (interaction). Finally, the tendency of case-control studies to show stronger associations than cohort studies suggests the possibility that some degree of recall bias and/or selection bias may have influenced the results of the former.

Critical issues in interpreting salt consumption with stomach cancer risk

Most epidemiological data suggest an association between salt intake and the development of stomach cancer. When interpreting these data, several issues must be considered.

Assessment of salt intake is difficult and prone to some potential biases. Many commonly used dietary assessment methods, such as food frequency questionnaires and diet records, have reported only

moderate reproducibility in epidemiological studies, and thus some misclassification of dietary intake is inevitable[79,80]. This may be particularly true of total salt intake, given its nearly ubiquitous addition to most processed foods. In addition, use of different salt assessment methods may lead to different conclusions. For example, both 24 h urinary excretion and 3 d dietary record methods for salt intake estimation were used in one Japanese ecologic study. Only urinary salt excretion level showed a strong correlation with stomach cancer mortality, while dietary salt was weakly and non-significantly correlated[21]. Although 24 h urine collection may be an optimal method in estimating routine salt intake, it is impracticable for a large-scale population study, especially a cohort study with long-term follow-up.

Another critical issue in interpreting salt consumption in relation to stomach cancer risk is the variation in the salt consumption levels across the population. To date, there is no standard method for salt intake categorization, and several studies have reasonably compared categories, such as quintiles for the application in the specific study population. Because average salt intake varies across different populations, salt consumption levels considered "high" in one study might be considered "low" in another study. For example, in a study conducted in Japan, subjects reporting once per week consumption of salted food were in the lowest exposure category[48]; in contrast, the subjects in a Turkish study reporting once per week were classified into the highest exposure level (defined as $\geqslant$1-2 times/week)[60]. Finally, in some studies[75], salted food levels were calculated from the sum of the scores of foods belonging to the food items (pickled vegetable + foods deep-boiled in soy sauce), making it impossible to compare effects at similar levels of consumption across studies.

Because of the complexity of diets, the traditional approach with a single nutrient may potentially be confounded by the interactions between food components that are likely to be interactive or synergistic[81]. It is possible that the increased risks in stomach cancer could be due to compounds other than salt in foods that were produced during the preservation process[56]. In East Asia, salted foods and sauces are also high in NO_3, a chemical carcinogen, which may either be added to the foods or synergize from amino acids during fermentation. Nitrite and salt may work at an early stage[82] in a synergistic fashion on stomach cancer carcinogenesis[17] that might cause the strong associations between highly salted foods and gastric cancer[67]. However, nitrite was not clearly related with stomach cancer risk[83] and its function may be influenced by other factors. For example, when lower salt intake was combined with higher NO_3 intake, stomach cancer mortality rates tended to be lower[17]. However, this might be explained by a higher intake of fresh fruits and vegetables, which are the major source of nitrate and also protect against cancer[84,85].

Moreover, it may be difficult to separate the effects of salt from other nutrients that may contribute to stomach cancer risk. The absence of adjustment for confounding factors (such as age, sex, smoking and dietary habit) can hamper the statistical estimation causing over- or underestimation of the real association between salt or salted food and stomach cancer. In Tables 1 and 2, few studies have controlled for dietary factors in their analyses of salt consumption, which makes it difficult to compare the different studies according to the dietary variables adjusted in the analysis. However, the study results that were adjusted by a wide range of potentially confounding variables, such as age, sex, *H pylori* infection, atrophic gastritis, medical history of peptic ulcer, family history of cancer, body mass index, diabetes, total cholesterol, physical activity, alcohol intake, smoking habit and other dietary factors[68], showed no difference from the crude results. Studies with adjustment for some or most of the above potential confounding factors[66,68,72,73] showed no systematically apparent differences from the studies with adjustment for a few or several confounders.

BIOLOGICAL MECHANISMS

Several mechanisms by which salt intake may increase stomach cancer risk have been postulated, although to date there has been no consistent conclusion. High dietary salt intake may potentiate the colonization of *H pylori*[86], a known risk factor for stomach cancer, through the increase of surface mucous cell mucin and decrease of gland mucous cell mucin[87]. At the molecular level, high dietary salt intake may potentiate *CagA* (*H pylori* gene) expression and enhance the ability of *CagA* to translocate into gastric epithelial cells and enhance the ability of *H pylori* to alter gastric epithelial cell function[15]. Another explanation for the potential effect of high salt intake in gastric carcinogenesis is that high dietary salt intake helps to change the mucous viscosity protecting the stomach, potentiate exposure to carcinogens such as N-nitroso compounds, and lead to cell death[88]. In addition, high salt intake can cause damage to, and inflammatory responses of, the gastric epithelium[14], which may increase epithelial cell proliferation as part of the repair process and increase the probability of endogenous mutations[89,90]. One mechanism of high salt action in gastric carcinogenesis has been considered to induce hypergastrinemia in *H pylori*-infected gerbils[87]. Gastrin itself may mediate epithelial cell growth in *H pylori*-colonized mucosa[91] and chronic hypergastrinemia can synergize with *Helicobacter* infection and lead to eventual parietal cell loss and progression to gastric cancer[92].

ANIMAL STUDIES OF SALT AND STOMACH CANCER

Most published animal studies focus on the relationship between gastric cancer and several important suspected carcinogens, salt, *H pylori*, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), and 4-nitroquinoline-1-oxide (NQO). In general, salt alone has no apparent

effect on the development of gastric carcinogenesis, but administration of salt in rats induced a concentration-dependent damage of surface mucous cell layer, and also increased replicative DNA synthesis[89]. Interestingly, a synergistic effect was observed when salt and other risk factors (*H pylori*, MNNG, NQO) were analyzed simultaneously. This conclusion was derived in animal experiments addressing gastric carcinogenesis from both the molecular level and tumor progression. At the molecular level, a high salt diet was associated with an elevation of anti-*H pylori* antibody titers, serum gastrin levels, and inflammatory cell infiltration in a dose dependent model in Mongolian gerbils infected with *H pylori*[87]. Similarly, in *H pylori* infected gerbils, a high-salt diet significantly up-regulated the expression of cyclooxygenase-2 (COX-2), and nitric oxide synthase (iNOS)[93]; the number of colony-forming units was also significantly higher. Dietary sodium chloride also produced a reduction in cell yield, and an increase in S-phase cell numbers that are the most susceptible to mutagenesis, which may possibly increase tumor incidence[90]. Several studies examined gastric tumor progression in mice infected with *H pylori* and administered with a high-salt diet, and all of these studies consistently demonstrate that a high-salt diet enhances the effects of *H pylori* infection, and consequently promotes the development of stomach cancers[88,94,95]. Additionally, a high-salt diet significantly increased gastric tumor incidence in those mice pre-treated with MNNG[14] or MNU[96], suggesting that salt and chemical carcinogens also exert a synergistic effect in the development of gastric carcinogenesis.

CONCLUSION

Most published epidemiological studies provide positive evidence for an association between salt or salted food consumption and stomach cancer risk, which was also supported by experimental studies[14,87,94,97]. The limitations of salt assessment in epidemiological studies may have attenuated the true effect of salt intake on stomach cancer risk, or even biased the results away from the null, in the reviewed ecological, case-control, and cohort studies. Ideally, dietary modification of salt intake, as well as eradication of *H pylori* infection, is a promising strategy for gastric cancer prevention throughout the world, especially in developing counties. However, the former strategy is more practical than the latter according to previous epidemiological studies. Future studies that address the association with salt and other dietary factors and the interactions between these factors in different aspects, e.g. molecular level, may help to shed light on the etiology of stomach cancer.

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ORIGINAL ARTICLES

Feasibility of confocal endomicroscopy in the diagnosis of pediatric gastrointestinal disorders

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Abstract

AIM: To evaluate the feasibility and utility of confocal laser endomicroscopy (CLE) in the description of normal gastrointestinal (GI) mucosa and in the diagnosis of GI disorders in children, in comparison to histology.

METHODS: Forty-four patients (19 female) median age 10.9 years (range 0.7-16.6 years) with suspected or known GI pathology underwent esophago-gastro-duodenoscopy (OGD) (n = 36) and/or ileocolonoscopy (IC) (n = 31) with CLE using sodium fluorescein and acriflavine as contrast agents. Histological sections were compared with same site confocal images by two experienced pediatric and GI histopathologists and endoscopists, respectively.

RESULTS: Duodenum and ileum were intubated in all but one patient undergoing OGD and IC. The median procedure time was 16.4 min (range 7-25 min) for OGD and 27.9 min (range 15-45 min) for IC. A total of 4798 confocal images were compared with 153 biopsies from the upper GI tract from 36 procedures, and 4661 confocal images were compared with 188 biopsies from the ileocolon from 31 procedures. Confocal images were comparable to conventional histology both in normal and in pathological conditions such as esophagitis, *Helicobacter pylori* gastritis, celiac disease, inflammatory bowel disease, colonic heterotopia, and graft versus host disease.

CONCLUSION: CLE offers the prospect of targeting biopsies to abnormal mucosa, thereby increasing diagnostic yield, reducing the number of biopsies, decreasing the burden on the histopathological services, and reducing costs.

Key words: Confocal laser endomicroscopy; Histology; Pediatric; Gastrointestinal mucosa; Gastrointestinal disorders

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INTRODUCTION

Modern endoscopy has recently seen the development of technological advances with the aim of increasing and optimizing diagnostic yield from the procedure. These have included video and magnification endoscopes[1]. Greater surface definition has been achieved with chromo-endoscopy, and recently, narrow-band imaging has allowed greater definition of vascular architecture[2-4]. However *in vivo* sub-surface pathology remained obscure to the endoscopist until the advent of confocal endomicroscopy, which affords magnification up to

1000 ×, and with sequentially deeper images from the epithelial surface to approximately 250 μm below the surface. This allows histological assessment of the *in vivo* gastrointestinal (GI) mucosal structure at the cellular and subcellular level[5,6]. In addition, this technique avoids crush artefacts from the grasp biopsy forceps and changes from histopathological processing.

The diagnosis of upper GI disorders in children depends to a great extent on endoscopy and subsequent histology of biopsy specimens[7]. Pathology such as gastroesophageal reflux disease (GERD)[8-10], eosinophilic esophagitis (EE)[11,12]; *Helicobacter pylori (H pylori)* gastritis[13,14], and celiac disease (CD)[15,16], in conjunction with various other investigative modalities have, as pivotal to their diagnosis, histological confirmation. Similarly, pediatric ileocolonic conditions such as inflammatory bowel disease (IBD)[17,18], familial adenomatous polyposis (FAP), graft versus host disease (GVHD)[19,20], and allergic colitis[21,22] necessitate a tissue diagnosis.

The aims of this study were to evaluate the feasibility and utility of confocal laser endomicroscopy (CLE) in the description of confocal features of normal GI mucosa and in the diagnosis of GI disorders in children.

MATERIALS AND METHODS

Patients

Forty-four patients with a potential diagnosis of GI pathology that required upper GI endoscopy and/or ileocolonoscopy as part of the clinical management were enrolled in the study. Written informed consent was obtained from parents and, where age and competency were appropriate, from each patient, before the examination. The study protocols were reviewed and approved by South Sheffield Regional Ethics Committee. Patient exclusion criteria were as follows: inability to give signed informed consent; age > 18 years; previous documented adverse reaction/allergy to sodium fluorescein or acriflavine hydrochloride; and non-correctable coagulopathy (PT > 14 s/platelet count < 90 000). The study was conducted between December 2005 and July 2007 at Sheffield Children's Hospital NHS Foundation Trust.

Indications for upper GI endoscopy alone included: children with suspected GERD; Barrett's esophagus; suspected peptic ulcer disease; suspected celiac disease based on raised anti-endomysial and tissue transglutaminase antibodies; and non-specific recurrent upper abdominal pain. Indications for ileocolonoscopy included: chronic diarrhea; presence of fecal blood; recurrent abdominal pain; weight loss; mutation of the APC gene; colonic heterotopia; and suspected GVHD.

Forty-four patients (19 female) with a median age of 10.9 years (range 0.7-16.6 years), and a median weight of 41.5 kg (range 8-97 kg) with suspected or known GI pathology were enrolled.

Patients undergoing ileocolonoscopy were admitted the previous day and had bowel preparation as for standard ileocolonoscopy. Patients undergoing upper GI endoscopy were admitted on the day of the procedure. All procedures occurred under general anesthesia, as is normal practice in our institution for pediatric GI endoscopy.

CLE

CLE involves the use of a highly miniaturized confocal microscope that has been incorporated into the distal tip of a flexible endoscope to allow *in vivo* microscopic examination of the gut mucosa. The confocal microscope uses a single optical fiber to deliver 488 nm laser light to the distal tip of the endoscope, where it is focused to a single diffraction-limited point within the tissue. The laser light excites fluorescent molecules within the tissue. Fluorescent light emanating from the specific point of focus is collected into the same optical fiber of the confocal microscope and delivered to the photodetector. Light emanating from outside the focally illuminated spot is not focused into the optical fiber and therefore, is geometrically rejected from detection. The focused point of laser light is scanned in a raster pattern across the field of view, and the intensity of the fluorescent signal returning to the detector from successive points is measured (12-bit digitization) to produce two-dimensional images that are *en face* to the tissue surface. By moving the microscope optics within the confocal microscope, the operator can dynamically adjust the imaging depth to allow microscopic imaging at and below the surface of the mucosa; hence each image is an optical section representing one focal plane within the specimen[5,23], and collection of multiple optical sections at successive depths results in true volumetric sampling of the tissue. As a three-dimensional volume is thus sampled, this can be thought of as a virtual biopsy.

The Pentax EC3870CILK endoscope has a 5-mm diameter miniaturized confocal microscope integrated into the distal tip of the endoscope. The diameter of the distal tip and insertion tube of the endoscope is 12.8 mm. In addition to the integrated confocal microscope, the distal tip also contains a color CCD camera which enables simultaneous confocal microscopy with standard video-endoscopy, air and water jet nozzles, two light guides, a 2.8-mm working channel, and an auxiliary water jet channel. During CLE, the laser delivers an excitation wavelength of 488 nm at a maximum laser output of 1 mW to the tissue (typically 300-700 μW). Confocal images can then be collected at either 1024 × 1024 pixels (0.8 frames/s) or 1024 × 512 pixels (1.6 frames/s). The optical sections have a 475 μm × 475 μm field of view, with a lateral resolution of 0.7 μm, axial resolution of 7.0 μm, and an imaging depth (z axis) range of 0-250 μm below the tissue surface, in 4-μm steps. The imaging depth below the tissue surface can be dynamically controlled by the operator. CLE magnifies images 1000-fold.

Contrast agents

Fluorescein sodium (FS) 10% and acriflavine hydrochloride (AH) 0.05% were used as contrast agents. FS is highly water-soluble and, on intravenous administration, rapidly diffuses in seconds from the

capillaries into the extra-vascular tissue. FS, when exposed to light of wavelength 465-490 nm (blue), emits light at longer wavelengths (520-650 nm, with the peak emission in the 520-530 nm green-yellow region)[24]. This enables visualization of microvessels, cells and connective tissue. However FS is not enriched in the nuclei of intestinal epithelial cells, and hence, the nuclei are not readily visible in the confocal images. To circumvent this limitation, AH (0.05%) is used topically to enrich the superficial nuclei and to a lesser extent the cytoplasm.

CLE was performed by a single experienced endoscopist (MT), who had completed the Mainz CLE training program prior to patient recruitment, using the confocal laser endomicroscope (EC3870CILK; Pentax, Tokyo, Japan). Ten to twenty milligrams Buscopan (hyoscine-N-butyl-bromide; Boehringer, Ingelheim, Germany) was given intravenously to limit peristaltic artefacts. Following duodenal or ileal intubation, 0.05-0.1 mL/kg of 10% FS was administered intravenously and flushed adequately with normal saline. AH (0.05%) was applied to the mucosa using a spray catheter at all sites undergoing confocal imaging.

CLE image acquisition was performed by placing the tip of the colonoscope in direct contact with the target tissue site. Using gentle suction to stabilize the mucosa, image acquisition and focal plane z-axis scanning depth was then actuated using two discrete hand-piece control buttons. Confocal images were sequentially obtained from a third part of the duodenum, gastric antrum and body, and distal and proximal esophagus in the upper GI tract, and ileum, cecum, ascending, transverse, descending and sigmoid colon, and rectum in the ileocolon region. Confocal images were acquired simultaneously with ongoing video endoscopic imaging. Same site mucosal specimens were obtained using standard biopsy forceps. The biopsy specimens were fixed in buffered formalin solution, embedded in paraffin wax, and serial sections were obtained and stained with hematoxylin and eosin (HE). The histological specimens from each site were compared with same-site confocal images jointly by the endoscopists and two experienced pediatric and GI histopathologists (MC, CE).

RESULTS

Twenty-three patients underwent both upper GI and ileocolonoscopy, while 13 had upper GI endoscopy and eight had ileocolonoscopy alone. The youngest patient 8 mo of age, with suspected GVHD, had proctoscopy alone. The duodenum at upper GI endoscopy and the terminal ileum at ileocolonoscopy were intubated in all patients, except for one who weighed 10 kg, in whom the pylorus and ileocecal valve were both too narrow to accept the confocal endomicroscope. The youngest and smallest patient to have full successful examinations up to a third part of the duodenum and terminal ileum was 18 mo old and weighed 11 kg. The procedure time was 7-25 min (median 16.4 min) for upper GI endoscopy, and 15-45 min (median 27.9 min) for ileocolonoscopy. A total of 153 pinch biopsies were taken from the upper GI tract from 36 procedures and 188 from the ileocolon from 31 procedures.

No complications or adverse effects occurred, except on one occasion when precipitation was observed in the peripheral venous line when fluorescein was injected immediately after neostigmine.

Upper GI tract

Thirty-six patients underwent upper GI endoscopy. The duodenum was intubated in all patients except one. A total of 4798 confocal images were obtained, which included 2010 from the duodenum, 1616 from the stomach and 1172 from the esophagus, and were compared with 153 biopsies.

On confocal imaging, the duodenal villi had a long, slender and finger-like appearance (Figure 1A) similar to that in histological specimens (Figure 1C). The single layer of brush border columnar epithelial cells interspersed with intraepithelial lymphocytes and goblet cells was clearly visible. Crypts were not normally visible except in the presence of villous atrophy.

The gastric pits or foveolae appeared as invaginations on the surface epithelium (Figure 1B and D). Each confocal image showed several evenly spaced such pits lined by columnar epithelium. The center of the pits appeared dark.

The esophagus was lined by non-keratinized squamous epithelium with polygonal epithelial cells. The nuclei of the epithelial cells were highlighted clearly following topical administration of acriflavine (Figure 1C and F). Furthermore, the capillary loops in the papillae were visible in deeper planes following subsurface optical sectioning, and surface to capillary distance could be measured as each level was deeper by 4 μm. This allowed assessment of GERD-like histopathology, given that papillary height was increased and epithelial surface to papillary tip (i.e. where capillary loops appeared on confocal endomicroscopy) distance was thereby shorter.

Lower GI tract

A total of 31 patients underwent ileocolonoscopy. Two patients had only proctoscopy, while total ileocolonoscopy was performed in the rest, with the terminal ileum intubated in all but one patient, who weighed 10 kg. A total of 4661 confocal images, which included 945 from the terminal ileum, 2919 from the colon and 797 from the rectum, were compared with 184 biopsies.

The confocal appearance of the normal ileum and colon in adults has been described previously[25]. The villi in the terminal ileum appeared similar to those in the duodenum. Colonic architecture on confocal imaging showed numerous evenly distributed crypts lined by columnar-shaped enterocytes (Figure 2A and B). The luminal openings of the crypts appeared as black holes in the horizontal axis. The mucin-containing goblet cells were readily identified and appeared dark. At deeper planes, the vessel architecture had a hexagonal, honeycomb pattern, which represented a network of capillaries that outlined the stroma surrounding the

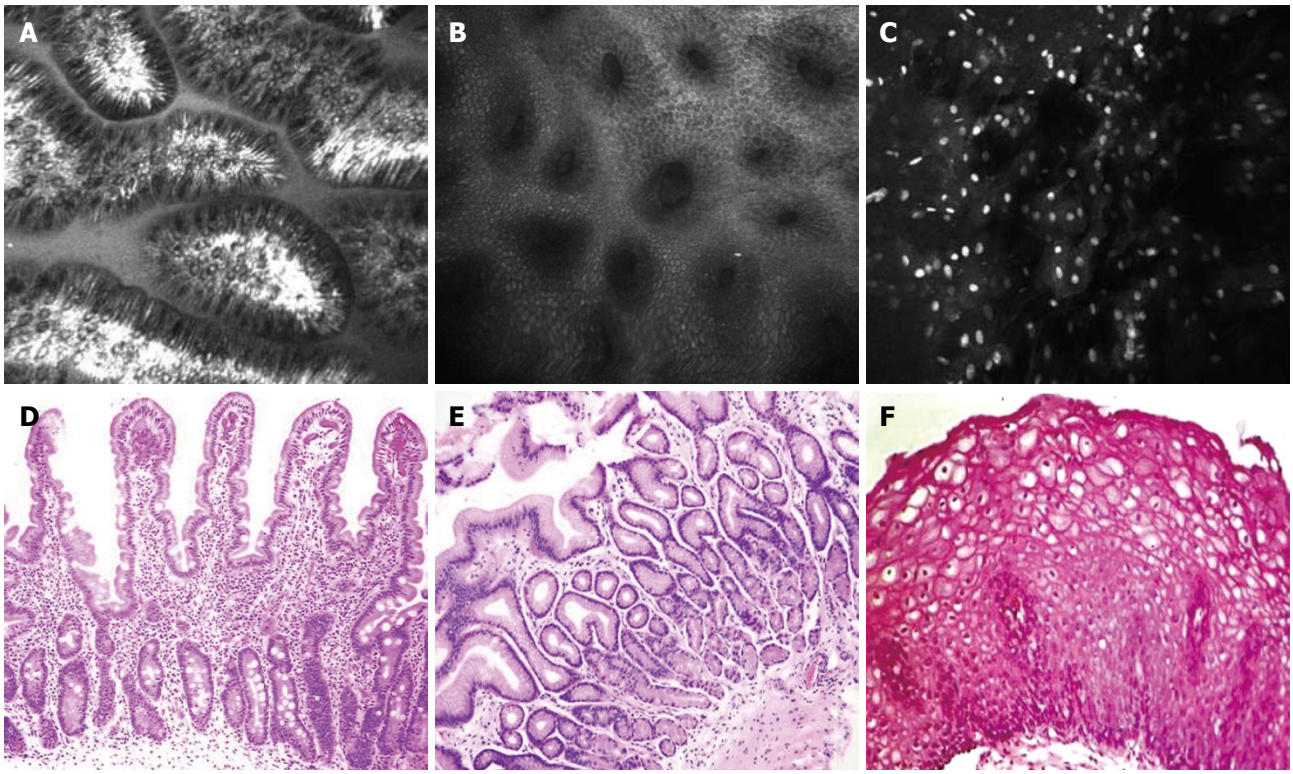

Figure 1 Comparison of confocal images with conventional histological images of the upper GI tract. A: Confocal image delineating the fine slender fingerlike projections of the duodenal villi; B: Confocal image showing gastric pits; C: Confocal image of non-keratinized squamous epithelium of the esophagus; D: Histological image of duodenum; E: Histological image of gastric antrum; F: Histological image of esophagus.

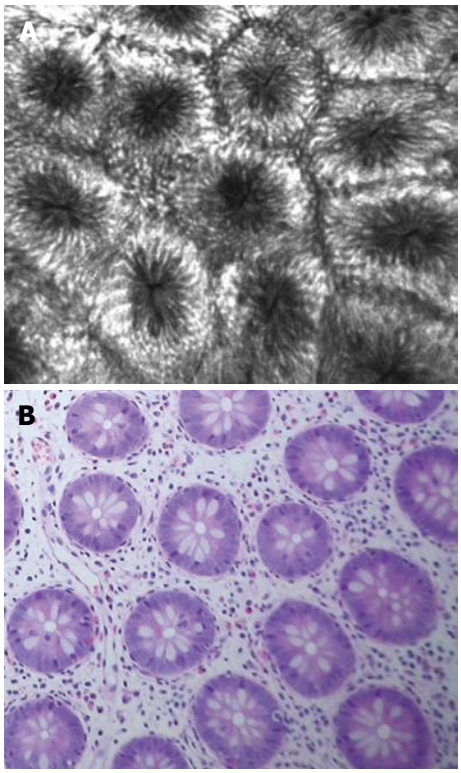

Figure 2 Comparison of confocal images with conventional histological images of the lower GI tract. A: Confocal image of normal colonic mucosa showing regularly spaced crypts; B: Comparative histological image.

luminal openings of the crypts. Individual red cells were also visible as black dots in the lumen of the capillaries.

Confocal imaging in GI pathology

Upper GI pathology: Two patients had histologically proven esophagitis. At CLE, capillary loops were visible at about 24 and 44 μm below the surface epithelial layer, which indicated the presence of papillae. In comparison, capillary loops were seen at a median of 72 μm (range 48-100 μm) from the surface of the esophageal mucosa in those without histologically proven esophagitis.

One patient with suspected *H pylori* upon endomicroscopy of the gastric antrum showed multiple focal lesions that resembled focal accumulation of *H pylori*, which was subsequently confirmed by Campylobacter-like organism test and histology. These lesions were demonstrated both on the surface of the epithelium and deeper in the crypt lumen.

Four patients had a histological diagnosis of celiac disease (Figure 3A and C). Three patients had Marsh type 3b with marked villous atrophy, increased intra-epithelial lymphocytes and crypt hyperplasia. CLE features (Figure 3B) in these patients were as follows: (1) increased basal width of villi; (2) gross distortion of the cellular architecture of the villous epithelium with loss of the honeycomb pattern; (3) damaged villous border; (4) "sticky" villi with inter-villous bridging; and (5) in-folding of villi. One patient had total villous atrophy. Confocal imaging revealed absence of villi with crypt hyperplasia (Figure 3D).

Lower GI pathology: Seven patients had a histological diagnosis of IBD, including three patients each with ulcerative colitis and Crohn's disease, and one with

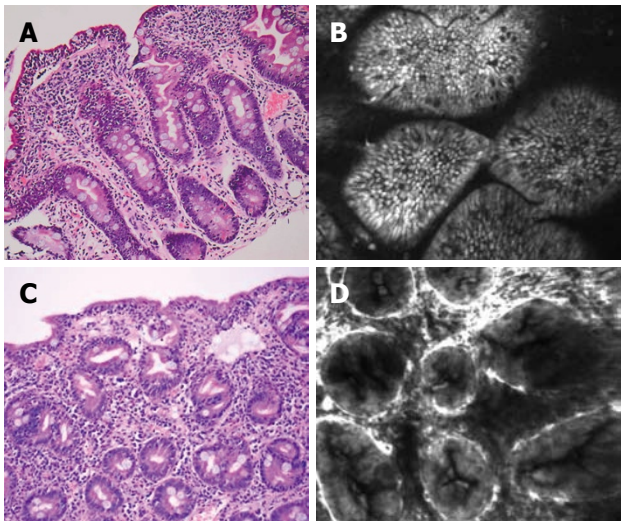

Figure 3 Comparison of confocal with conventional histology in celiac disease. A: Histological image of celiac disease, Marsh type 3b; B: Comparative confocal image; C: Histological image of celiac disease, Marsh type 3c; D: Comparative confocal image.

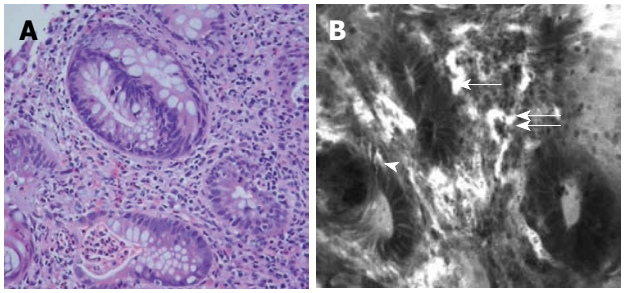

Figure 4 Comparison of confocal with conventional histology in ulcerative colitis. A: Histologic image in Ulcerative colitis; B: Comparative confocal image showing bifid crypt (arrow), crypt destruction (arrow head), tortous vessels (double arrows).

indeterminate colitis. Features of IBD seen on confocal imaging included bifid crypts, crypt distortion and destruction, crypt abscess/cryptitis goblet cell depletion and inflammatory cell infiltration, enlarged tortuous vessel architecture (Figure 4B), and comparable to histology (Figure 4A).

Other lower GI pathologies: Two patients with suspected GVHD following bone marrow transplantation underwent proctoscopy. Apoptotic nuclei were visualized during confocal imaging. This was confirmed on histology of biopsy specimens. A rare case of colonic heterotopia that presented with persistent diarrhea and had large tracts of abnormal looking mucosa on endoscopy, showed squamous, gastric and small-intestinal mucosal features on confocal imaging. Histology confirmed the presence of aberrant mucosa.

DISCUSSION

A definitive diagnosis of GI disorders in children usually requires GI endoscopy and histology of biopsy tissue. Technological innovations have led to the development of chromo-endoscopy, for which dyes such as methylene blue and indigo carmine are used to aid localization of lesions, and magnifying endoscopy has enabled visualization of surface structures at approximately × 100 magnification. In adults, several studies have validated these techniques in differentiating neoplastic from non-neoplastic lesions[26-29], diagnosis of neoplastic lesions in flat and depressed lesions in the colorectum, and in cancer surveillance in patients with long-standing ulcerative colitis[30,31]. Confocal endomicroscopy is a newly developed tool that enables surface and subsurface imaging of living cells in the mucosa during ongoing endoscopy. It offers the combination of video endoscopy and confocal endomicroscopy, which uniquely provides *in vivo* histology and what might be termed a virtual biopsy[6]. The confocal endomicroscopy images obtained are in a single optical plane parallel to the surface of the tissue. Collection of multiple optical sections at successive depths allows detailed visualization of successive tissue layers, and allows sampling of a three-dimensional volume of tissue. This is in contrast to conventional histology in which the tissue is sectioned vertically, making it possible to see all the tissue layers in one view using a bench top light microscope. Hence, it is pertinent that confocal images require comparison with similarly sectioned histological images. The endoscopist also requires training in normal and abnormal microscopic anatomy, which takes time. Also a certain amount of training in using the endomicroscope and interpreting the image data is necessary.

In this study, the feasibility of CLE in the diagnosis of GI disorders was determined in children as young as 8 mo of age and as light as 10 kg, for the first time. Confocal findings of normal GI mucosa are described. In addition, confocal features in conditions such as pediatric manifestations of GERD, *H pylori* gastropathy, celiac disease, IBD, GVHD, and colonic heterotopia were illustrated.

The tantalizing prospect of targeted biopsies or even a biopsy-free endoscopic procedure in the diagnosis of childhood GI disorders arises, with obvious potential benefits in terms of avoidance of biopsy-associated complications, and diminution of the considerable histological burden that this patient cohort places on already over-stretched histopathological services, along with the prospect of considerable associated cost savings.

COMMENTS

Background

Histology of biopsy specimens has a major role in the definitive diagnosis of pediatric gastrointestinal (GI) disorders, but is subject to changes from crush artefacts and processing, in addition to an inherent delay in diagnosis. Confocal laser endomicroscopy (CLE) is a recent development that enables surface and subsurface imaging of living cells *in vivo* at × 1000 magnification.

Research frontiers

The relatively new tool of CLE has been used in the assessment and *in vivo* diagnosis of various GI disorders in adults, such as Barrett's esophagus, esophageal, gastric and colorectal cancers, and inflammatory bowel disease and other forms of colitis.

Innovations and breakthroughs

This is believed to be the first study to assess this innovation in the diagnosis of pediatric GI disorders. This study confirmed the feasibility and diagnostic

reliability of CLE for the *in vivo* diagnosis of a wide range of GI disorders in childhood.

Applications

This study provides a basis for future studies on the use of this advanced diagnostic technique in providing a real-time diagnosis during endoscopy in children.

Peer review

This was a well-conducted study, which shows that it is feasible to use confocal endomicroscopy in the diagnosis of childhood GI disorders.

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ORIGINAL ARTICLES

Intestinal microflora molecular markers of spleen-deficient rats and evaluation of traditional Chinese drugs

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Abstract

AIM: To find a rapid and efficient analysis method of gastrointestinal microflora in Pi-deficient (spleen-deficient) rats and to evaluate traditional Chinese drugs.

METHODS: Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) based assay was performed to examine changes of intestinal microflora in two Pi-deficienct animal models and to evaluate the efficacy of four traditional Chinese drugs as well as a probiotic recipe and another therapy in Pi-deficient rats.

RESULTS: A molecular marker was identified for Pi-deficiency in rats. The pharmacodynamic evaluation system, including identified molecular markers (net integral area and abundance of DNA bands), Shannon's index for diversity of intestinal microflora, and Sorenson's pairwise similarity coefficient, was established. The four major clinical recipes of traditional Chinese drugs for Pi-deficiency in rats, especially at their medium dose (equivalence to the clinical dose), produced more pronounced recovery activities in Pi-deficient rats, while higher doses of these recipes did not show a better therapeutic effect but some toxic effects such as perturbation deterioration of intestinal microflora.

CONCLUSION: Both fingerprint analysis and identified marker can show Pi-deficiency in rats and its difference after treatment. The identified molecular marker may be applied in screening for the active compounds both in relative traditional Chinese drugs and in pharmacodynamic study of Pi-deficiency in rats.

Key words: Pi-deficiency; Enterobacterial repetitive intergenic consensus-PCR; Traditional Chinese medicine

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INTRODUCTION

Pi-deficiency (spleen deficiency), a common clinical syndrome in traditional Chinese medicine (TCM), is described as symptoms such as epigastralgia, flatulence after meal, lack of appetite, wilted complexion, loose stool, lassitude, fatigue, *etc.* The "Pi" here is the Chinese spelling of "spleen" in TCM, which relates to the functions of digestion, absorption and nutrition, differs from the "spleen" in Western medicine that belongs to the blood and immune systems. Pi-deficiency in TCM is one of the most common digestive diseases and usually the patients' equilibrium of gastrointestinal microflora is broken, which plays an important role in the growth, development and performance of the host[1]. Therefore, more clinical interests are arising in monitoring changes of intestinal microflora in intestinal disease and its consequent treatment with TCM therapies. It has been found that some traditional Chinese drugs have curative effects on Pi-deficiency by regulating the equilibrium of intestinal microflora and therefore promote the recovery of Pi-deficiency[2-4].

However, methods of monitoring the intestinal flora are quite limited, not only because of the complexity of

its constitution, but also the difficulty in culturing most gastrointestinal bacteria *in vitro*. Recent development in molecular biology techniques provides various possibilities of illustrating microbial biodiversity without *in vitro* culture of bacteria[5-6]. Enterobacterial repetitive intergenic consensus (ERIC) sequences are non-coding sequences of highly conserved 127 bp that are repeated multiple times through the genome of most bacterial species[7]. Variation in the number and location of ERIC sequences between different populations of microbes will result in differences between strains in the number and size of PCR products by ERIC primers. Based on this, ERIC-PCR has been used to investigate the diversity of bacteria[8-11].

In this study, we introduced ERIC-PCR fingerprinting in study of Pi-deficiency syndrome, used molecular markers to detect changes of intestinal microflora in Pi-deficient rats, and evaluated the therapeutic effects of traditional Chinese drugs.

MATERIALS AND METHODS

Animals

Wistar rats (200 ± 20 g) of either sex were obtained from the Experimental Animal Centre of Shenyang Pharmaceutical University (Shenyang, China). The rats were kept under standard environmental conditions with free access to rodent diet and water. All animal experiments were performed in accordance with the Guidelines for Use of Experimental Animals established by Shenyang Pharmaceutical University.

Plant materials

Plant materials including *Radix* and *Rhizoma Rhei*, *Folium Sennae, etc,* used in the study, were purchased from a local TCM apothecary in Shanghai, China (Table 1), and identified by Dr. Meng-Yue Wang, Department of Pharmacognosy, School of Pharmacy, Shanghai Jiao Tong University.

Preparation of traditional Chinese drug decoctions

One hundred milliliters aqueous decoction was prepared with 100 g of each crude *Radix, Rhizoma Rhei* and *Folium Sennae*. For the preparation of decoction of traditional Chinese drug recipes, the crude drugs were mixed first according to the ratio as prescribed, and then decocted 3 times in 10 volumes of distilled water for 30 min, finally the solution was filtered and concentrated. The ratios and concentrations are shown in Table 1.

Induction of Pi-deficiency in rats and treatment with traditional Chinese drugs

Rats were randomly divided into 16 groups (n = 8). Rats in group 1 received distilled water only (10 mL/kg, *po*) during the whole experiment. Rats in groups 2-16 were intragastrically given *Radix* and *Rhizoma Rhei* extract, 10 mL/kg, twice a day for the first 10 d to induce Pi-deficiency[12]. Rats in group 2 (model group) received distilled water only, once a day for 10 d. Rats in group 3 received Entrocoordinatibiogen (16.2 mg/kg, *po*), once a day for 10 d. Rats in group 4 received Banxia Houpu Tang (Decoction of Pinellia and Magnolia Bark, 4.3 g crude drug/kg, *po*), once a day for 10 d. Rats in groups 5-7 were treated with Si Junzi Tang (Decoction of Four Noble Drugs, 1.2, 3.5 and 10.5 g of each crude drug/kg, *po*), once a day for 10 d. Rats in groups 8-10 received Lizhong Tang (Decoction for Regulating the Function Of Middle Jiao, 1.8, 5.4 and 16.2 g of each crude drug/kg, *po*), once a day for 10 d. Rats in groups 11-13 received Buzhong Yiqi Tang (Decoction for Regulating the Function Of Middle Jiao and Supplementing Qi, 1.6, 4.8 and 14.4 g of each crude drug/kg, *po*), once a day for 10 d. Rats in groups 14-16 received Yiwei Tang (Decoction for Nourishing the Stomach, 1.9, 5.8 and 17.4 g of each crude drug/kg, *po*), once a day for 10 d. Banxia Houpu Tang is a recipe for tussis but not for Pi-deficiency. Si Junzi Tang, Lizhong Tang, Buzhong Yiqi Tang and Yiwei Tang are commonly used clinical recipes for Pi-deficiency. Another experiment was performed as described above, except that 10 mL/kg *Folium Sennae* was given instead of *Radix and Rhizoma Rhei* to induce Pi-deficiency[13,14].

Sample collection and total DNA extraction

Three or four pieces of fecal pellets (about 1 g) per rat were directly collected from the anus into sterile plastic tubes and stored at -20℃ immediately. Fecal pellets were collected 5 d before induction of Pi-deficiency and then every two days.

Total DNA was isolated from the fecal samples as previously described[10] with some modifications. Each sample (0.2 g) was suspended in 1 mL sterile 0.05 mol/L PBS (pH 7.4) followed by vortexing for 5 min in a 2 mL tube. The suspension was centrifuged at 200 × g for 6 min and the supernatant was transferred to a new tube. Then 1 mL sterile PBS was added to the pellets and vortexed for 5 min, the suspension was centrifuged and the supernatant was transferred to the new tube as well. Combination of the two sets of supernatant was then centrifuged at 300 × g for 6 min to remove coarse particles. The cells in the supernatant were collected and washed twice with PBS by centrifuging at 10000 r/min for 6 min. The washed cell pellets were resuspended in 300 μL of solution Ⅰ containing 150 mmol/L NaCl, 50 mmol/L Na_2EDTA (pH 8.0). The suspension was gently mixed with 100 μL lysozyme solution (100 mg/mL) and 20 μL RNase (10 mg/mL), pre-warmed in 37℃ water bath for 30 min and then combined with 300 μL of solution Ⅱ containing 100 mmol/L NaCl, 50 mmol/L Tris base (pH 8.0). The cell suspension was gently mixed with 100 μL of 10% SDS and 50 μL of 20% PVP, and incubated on ice for 5 min. DNA was then purified by sequential extraction with Tris-equilibrated phenol and chloroform-isoamyl alcohol (v/v/v, 25:24:1), and chloroform isoamyl alcohol (v/v, 24:1) followed by precipitation with 2 volumes of ethanol and 50 μL of 3 mol/L sodium acetate. DNA was collected by centrifugation and washed once with 70% ethanol,

Table 1 Clinical recipes of traditional Chinese drugs used in this study

Prescription	Composition	Dose (g crude plants/kg)	Effect[a]
Banxia Houpu Tang	*Rhizoma Pinelliae*, *Poria*, *Cortex Magnoliae Officinalis*, *Folium Perillae*, *Rhizoma Zingiberis Recens* (4:4:3:3:2)	4.3 (clinical dose)	N↓, A↓, C↓, H↓
Si Junzi Tang	*Radix Ginseng*, *Rhizoma Atractylodis Macrocephalae*, *Poria*, *Radix Glycyrrhizae* (10:9:9:6)	1.2 (triplicate of clinical dose)	N↓[1], A↓[1], C↑, H↑
		3.5 (clinical dose)	N↓[1], A↓[1], C↑[1], H↑
		10.5 (triplication of clinical dose)	N↓[1], A↓, C↑, H↓
Lizhong Tang	*Radix Codonopsis*, *Rhizoma Atractylodis Macrocephalae*, *Rhizoma* Zingiberis, *Radix Glycyrrhiza preparata* (1:1:1:1)	1.8 (triplicate of clinical dose)	N↓, A↓, C↑, H↑
		5.4 (clinical dose)	N↓[1], A↓[1], C↑, H↑
		16.2 (triplication of clinical dose)	N↓[1], A↓, C↑, H↑
Buzhong Yiqi Tang	*Radix Astragali*, *Radix Ginseng*, *Radix Angelicae Sinensis*, *Rhizoma Atractylodis Macrocephalae*, *Radix Glycyrrhiza preparata*, *Radix Bupleuri*, *Rhizoma Cimicifugae*, *Pericarpium Citri Reticulatae* (6:1:1:1:3:2:2:2)	1.6 (triplicate of clinical dose)	N↓[1], A↓[1], C↑, H↑
		4.8 (clinical dose)	N↓[1], A↓[1], C↑[1], H↑
		14.4 (triplication of clinical dose)	N↓[1], A↓, C↑, H↓
Yiwei Tang	*Radix Glehniae*, *Raidix Ophiopogonis*, *Rehmannia Dride Rhizome*, *Rhizoma Polygonati Odorati*, rock candy (3:3:3:1:3)	1.9 (triplicate of clinical dose)	N↓[1], A↓[1], C↑, H↓
		5.8 (clinical dose)	N↓[1], A↓[1], C↑, H↓
		17.4 (triplication of clinical dose)	N↓, A↓, C↑, H↓

[a]N: Net area of 380 bp; A: Abundance of 380 bp; C: Sorenson's pairwise similarity coefficient (Cs); H: Shannon's index (H'); ↑: Increase; ↓: Decrease; [1]: Significant ($P < 0.05$).

air dried and dissolved in 50 μL of sterile distilled water. The DNA was checked for integrity first by electrophoresis analysis on 1% agarose gel (compared with size-known Hind Ⅲ digested bacteriophage λ DNA), and then quantified.

ERIC-PCR

ERIC-PCR was performed on a MJ Research PTC-100 thermal cycler (MJ Research, Inc., Waltham, USA) using the ERIC primers (ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3', ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3')[7]. The reaction system was optimized and determined with orthogonal array design and statistic analysis method as previously described[15]. PCR consisted of 2.5 μL 10 × buffer, 200 μmol/L dNTP, 2.5 mmol/L Mg^{2+}, 0.4 μmol/L primer, 1U HotstarTaq DNA polymerase and 2 μL DNA template (or correspondingly 2 μL sterile distilled water in controls) in a total 25 μL volume. PCR conditions were as follows: an initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing at 95℃ for 50 s, annealing at 49℃ for 30 s, at 46℃ for 30 s, extension at 72℃ for 3 min, and a final extension at 72℃ for 9 min. PCR products were separated by electrophoresis on 2% agarose gel (Agarose LE, Mdbio, Inc.) containing 0.5 μg/mL ethidium bromide and observed under UV light by Tannon GIS2010 Image System Ver. 3.73 (Tanon, Inc., Shanghai, China). The size and quantity of the amplified fragments were determined using 1 kb plus DNA makers (Tiangen, Inc., Beijing, China).

Statistical analysis of ERIC-PCR fingerprint

ERIC-PCR profiles were analyzed using the Gel Compare function of Tannon GIS2010 Image System Ver. 3.73 and transformed to data sets by taking into account the relative square root of the area under each PCR peak and abundance of each peak. Similarities between samples and their temporal stability were determined by calculating Sorenson's pairwise similarity coefficient (Cs), which is commonly used to compare the species composition of different ecosystems. Two identical profiles create a value of 100%, whereas two completely different profiles result in a value of 0%.

$$Cs\ (\%) = (2 \times j)/(a+b) \times 100\%$$

where 'a' is the number of total bands in the ERIC-PCR pattern for one sample, 'b' is the number for the other, and 'j' is the number of the common bands shared by both samples[16].

Shannon's index (H'), which originally refers to the community richness, was also employed to measure the distribution of PCR bands in our study. We used it to describe the quantitative difference in intestinal microflora under different conditions, although each ERIC-PCR band does not have to stand for one individual bacterial species.

$$H' = -\sum(Pi)(\ln Pi)$$

where P*i* is the relative abundance of each band, calculated as the proportion of the *i*th band in the fringerprint[16-18].

Results were described as mean ± SE. The statistical significance ($P < 0.05$) of difference between means was determined using paired-samples *t* test or ANOVA with SPSS version 11.5 (SPSS Inc., Chicago, USA), where appropriate.

RESULTS

ERIC-PCR fingerprint of intestinal microflora in naive rats

ERIC-PCR profiles of total fecal DNA were obtained for samples collected from naive rats before induction. Repeats of fingerprints showed that there were 9-12 fragments ranging 120-3000 bp with various intensities. There was a considerable variation of ERIC-PCR profiles between individual rats, in which only approximately 50% similarity was seen (Figure 1A). However, samples collected on different days from the same rat showed much a better consistency, with a similarity (Cs) ranging 63%-88% (data not shown). The occurrence of each fragment was calculated using Tannon GIS2010 Image System. Two fragments (590

Table 2 Occurrence and preliminary biomarkers of intestinal microflora ERIC-PCR fingerprinting in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* (n = 100)

Fragments	Occurrence (%)		Net integral area			Abundance		
	Before administration (healthy)	After administration (Pi-deficiency)	Before administration (healthy)	After administration (Pi-deficiency)	Range	Before administration (healthy)	After administration (Pi-deficiency)	Range
590 bp	79	53	851.65 ± 68.00	385.76 ± 37.63[a]	Decrease 55%	18.49 ± 1.36	11.69 ± 1.05[a]	Decrease 37%
380 bp	33	94	98.61 ± 18.09	563.64 ± 32.94[a]	Increase 470%	2.74 ± 0.58	19.16 ± 1.30[a]	Increase 590%
300 bp	75	10	604.93 ± 46.67	70.87 ± 23.12[a]	Decrease 88%	12.70 ± 0.94	2.31 ± 0.71[a]	Decrease 82%

Paired samples t test, [a]$P < 0.05$ *vs* before administration.

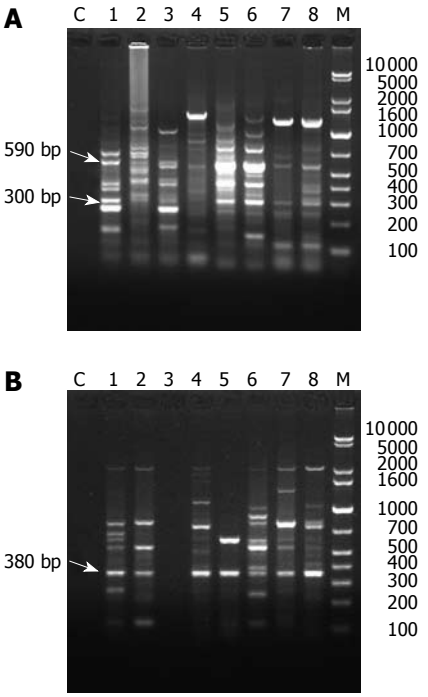

Figure 1 ERIC-PCR fingerprinting of intestinal microflora from feces of 8 out of 128 rats before (A) and after (B) Pi-deficiency induced by *Radix* and *Rhizoma Rhei*. C: Water administration; M: Ladder; lanes 1-8: rats. Rat No. 3 died of trauma after uptake of *Radix* and *Rhizoma Rhei*.

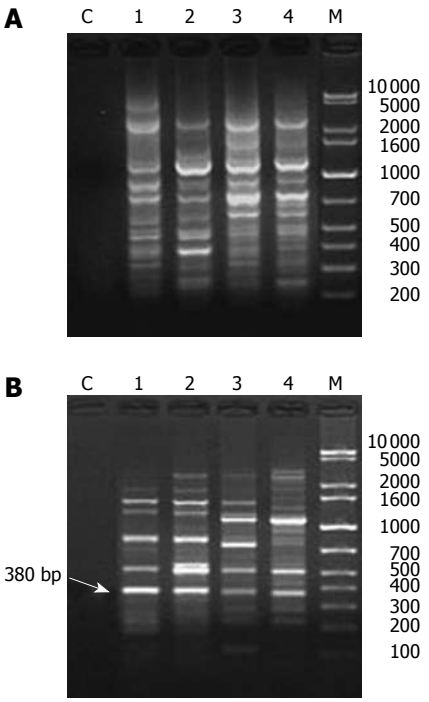

Figure 2 ERIC-PCR fingerprinting of intestinal microflora from feces of 4 out of 128 rats before (A) and after (B) Pi-deficiency induced by Folium Sennae. C: Water administration; M: Ladder; lanes 1-4: rats.

bp and 300 bp) showed a higher occurrence of 83% and 74% respectively (occurrence > 70%) among the fingerprints of 128 rats, indicating that these two predominant bands are likely to be populations-associated naive rat gastrointestine.

ERIC-PCR fingerprint of intestinal microflora in Pi-deficient rats

Symptoms of Pi-deficiency[1-4], including humped back, narrow eyes, watery stools, listlessness, lack of appetite and weight loss[14] occurred in the rats that received *Radix* and *Rhizoma Rhei* or *Folium Sennae*. A remarkable difference in ERIC-PCR profile was found between rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* or *Folium Sennae* and normal rats (Figures 1 and 2). Shannon's index (H') of the rat Pi-deficiency model (1.77 ± 0.03, n = 200) was significantly lower than that of normal ones (2.02 ± 0.02, $P < 0.05$, n = 200), indicating that altered profiles and lesser diversities of ERIC-PCR fingerprints are in the status of Pi-deficiency. The similarity (Cs) of ERIC-PCR fingerprints of the same rat before and after Pi-deficiency induction decreased to approximately 39% in groups 2-16, whereas 62% in control group that received distilled water only ($P < 0.05$), suggesting that the constitution of intestinal bacterial community in Pi-deficiency rats is significantly different from that in normal rats.

Molecular markers of intestinal microflora in rats with Pi-deficiency induced by Radix and Rhizoma Rhei

Analysis of ERIC-PCR profiles for 100 rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* implied that three fragments (590, 380 and 300 bp) showed that *Radix* and *Rhizoma Rhei* administration can induce significant changes in abundance and band net integral area ($P < 0.05$), as well as the occurrence of those fragments (Table 2). The 590 bp and 300 bp fragments, especially the 300 bp fragment, were shown in most normal rats, but in much fewer rats after Pi-deficiency induction (Figure 1B). Different from the 590 bp and

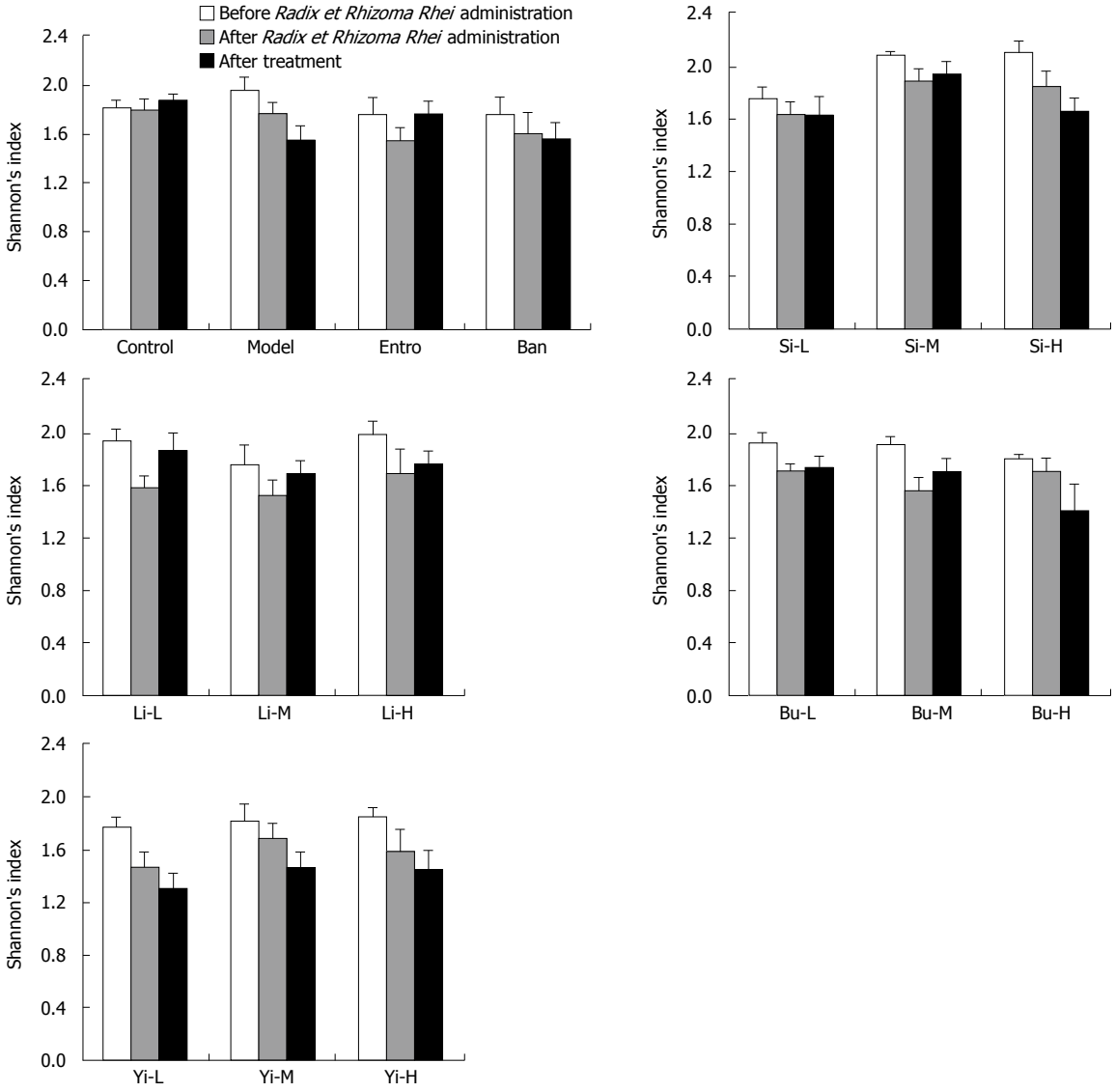

Figure 3 Shannon's index of ERIC-PCR fingerprintings of Pi-deficient rats before and after TCM treatment. Pi-deficiency was induced by *Radix* and *Rhizoma Rhei* first in all groups except for the control group (Group 1) that received distilled water. Control: Group 1 received distilled water in both inducement and treatment phases. Model: Group 2 received *Radix et Rhizoma Rhei* but distilled water during treatment; Entro: Group 3 received Entrocoordinatibiogen during treatment; Ban: Group 4 received decoction of *Ban-xia-hou-pu-tang* during treatment; Si-L, Si-M, Si-H: Groups 5-7 received low, middle and high doses of *Si-jun-zi-tang*, respectively; Li-L, Li-M, Li-H: Groups 8-10 received low, middle and high doses of *Li-zhong-tang*, respectively; Bu-L, Bu-M, Bu-H: Group 11-13 received low, middle and high doses of *Buzhong Yiqi Tang*, respectively; Yi-L, Yi-M, Yi-H: Groups 14-16, received low, middle and high doses of *Yi-wei-tang*, respectively.

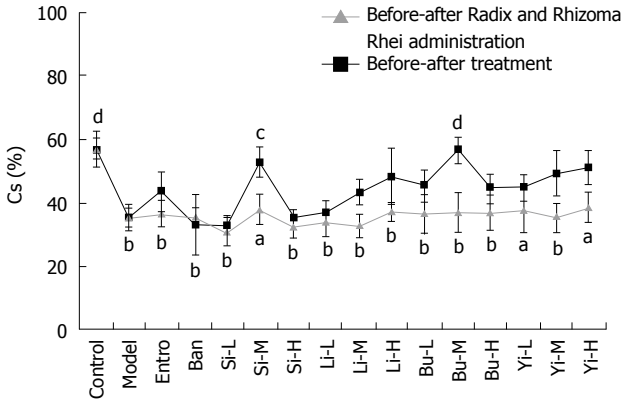

Figure 4 Similarity coefficient (Cs) for ERIC-PCR fingerprintings of Pi-deficient rats before and after treatment. Samples were the same as in Figure 3. Gray: Cs before and after *Radix* and *Rhizoma Rhei* uptake; Black: Cs before and after treatment. Statistical significance of differences was calculated by One-Way ANOVA. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group.

300 bp fragments, the 380 bp fragment was not seen in normal rats, but in most Pi-deficient rats, indicating that *Radix* and *Rhizoma Rhei* administration can induce great changes in the proportion of individual bacterial species. The rest fragments were randomly detected in either normal or Pi-deficient rats, with no correlation between the presence of fragments and Pi-deficiency. Therefore, these fragments (590, 380 and 300 bp) were selected as preliminary biomarkers for intestinal microflora ERIC-PCR fingerprints of rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei*.

In order to further identify the optimal biomarker of ERIC-PCR fingerprints for rats with Pi-deficiency, the profile of preliminary biomarkers (590, 380 and 300 bp) in different groups of Pi-deficient rats that received TCM treatment was also investigated. As a result, 4 TCM recipes restored the net integral area and abundance of 380 bp fragment to a certain extent. However, changes

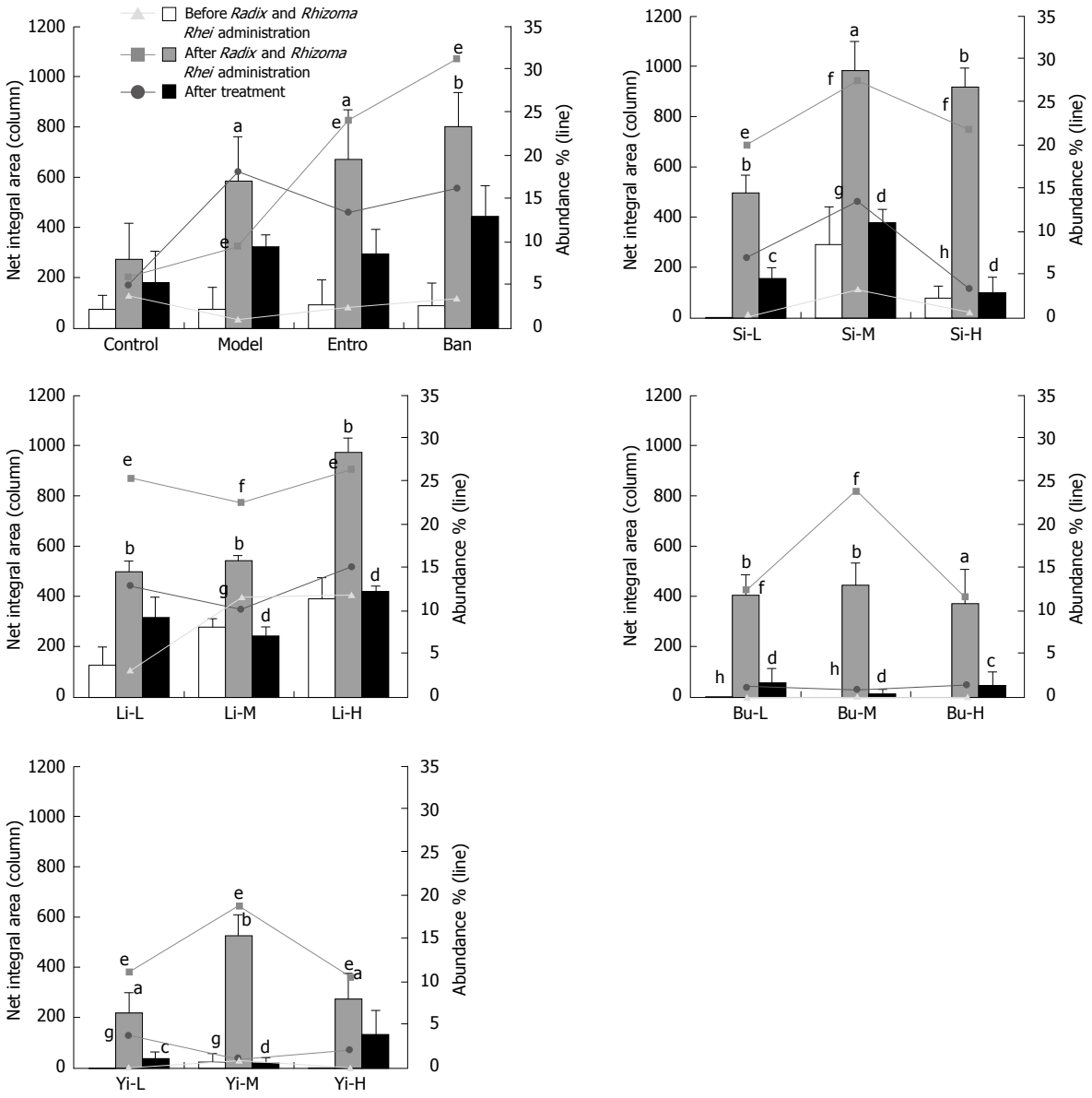

Figure 5 Net integral area and abundance of the 380 bp fragment in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* and after treatment. Columns: Net integral area of the 380 bp fragment; Lines: Abundance of 380 bp fragment. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* healthy condition; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* Pi-deficiency status; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* healthy condition; $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* Pi-deficiency status. Samples were the same as in Figure 3.

in 590 bp and 300 bp fragments were not as significant as in 380 bp fragment (data not shown). Given the data above, the 380 bp fragment was identified as the biomarker of ERIC-PCR fingerprints for rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei*, the net integral area and abundance of the 380 bp fragment could therefore be used as parameters to evaluate the therapeutic effects of TCM on Pi-deficiency.

Additionally, in 7 out of 12 Pi-deficient groups that received TCM recipes, The Shannon's index (H') of ERIC-PCR fingerprints was restored (Figure 3). A similar trend of the similarity (Cs) of ERIC-PCR fingerprints was seen in those groups that received TCM recipes (Figure 4), indicating that Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs) can also be considered as biomarkers for Pi-deficiency and used to evaluate the therapeutic effects of TCM on Pi-deficiency.

Evaluation of therapeutic effects of TCM recipes on P1-deficiency induced by Radix and *Rhizoma Rhei using the identified biomarkers*

As shown in Figures 3-5 and Table 1, Si Junzi Tang reduced the net integral area and abundance of the 380 bp fragment and increased the Sorenson's pairwise similarity coefficient (Cs) in a dose-dependent manner. The effects were most significant at the dose of 3.5 g crude drug/kg. However, the Shannon's index (H') increased after treatment with Si Junzi Tang at the dose of 3.5 g crude drug/kg, but decreased after treatment with Si Junzi Tang at a higher dose of 10.5 g crude drug/kg.

The net integral area and abundance of the 380 bp were significantly different before and after treatment with Lizhong Tang at the dose of 5.4 g. The Sorenson's pairwise similarity coefficient (Cs) increased in a dose-dependent manner and the Shannon's index (H') also increased at the three doses with no significant

difference.

Moreover, Buzhong Yiqi Tang also significantly decreased the 380 bp fragment and increased the Sorenson's pairwise similarity coefficient (Cs) in a dose-dependent manner, in which the maximal and significant effects were shown at the dose of 4.8 g crude drug/kg. Buzhong Yiqi Tang increased and decreased the Shannon's index (H') at the doses of 1.6 and 4.8 g crude drug/kg, and 14.4 g crude drug/kg, respectively.

The net integral area and abundance of the 380 bp were decreased after treatment with Yiwei Tang, indicating that the effect is statistically significant at the dose of 1.9/5.8 g crude drug/kg. Yiwei Tang increased the Sorenson's pairwise similarity coefficient (Cs) in a dose-dependent manner. However, the Shannon's index (H') was lower than that before treatment.

The H' and Cs values as well as the net integral area and abundance of the 380 bp fragment were decreased in rats that received water, which might be due to the long-term Pi-deficiency. A similar trend was seen in the group that received Banxia Houpu Tang. Entrocoordinatibiogen increased the Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs) ($P < 0.05$), but had no significant effects on the net integral area and abundance of the 380 bp fragment.

Evaluation of therapeutic effects of TCM on Pi-deficieny induced by Folium Sennae

The four biomarkers (H', Cs, net internal area and abundance of the 380 bp fragment) that were identified in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* were also proved to be valid for *Folium Sennae* induced Pi-deficiency. The four recipes (Si Junzi Tang, Lizhong Tang, Buzhong Yiqi Tang and Yiwei Tang) for Pi-deficiency significantly reduced the net integral area and abundance of the 380 bp fragment at smaller and medium doses, but significantly increased the Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs) (data not shown).

DISCUSSION

This study reported the changes of intestinal microflora in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* or *Folium Sennae*. ERIC-PCR fingerprinting system is highly reproducible when it is used to examine the status of intestinal microflora in rats. In this study, fingerprints of the Sorenson's pairwise similarity coefficient (Cs) from the same DNA extraction were over 95%, respectively.

The dominating intestinal microbial population may vary in subjects due to changed physiological conditions. The replicates (collected on different days) of PCR fingerprints from the same rat showed a high reproducibility (Cs > 75%). However, this value decreased to 57% after water administration, indicating that water administration can affect the intestinal physiology after intragastric operation. These results indicate that ERIC-PCR is a sensitive tool for examining the structure of fecal bacterial community.

Entrocoordinatibiogen (Shenyang No. 1 Pharmaceutical Factory, Shenyang, China) consisting of bacillus licheniformis, a kind of probiotics and a biotherapeutic agent modulating microdysbiosis of intestine, is used in treatment of acute bacillary dysentery. Based on the analysis of ERIC-PCR intestinal microflora molecular markers, the rats that received Entrocoordinatibiogen had a certain extent of recovery as indicated by the increased Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs). However, neither the net integral area nor the abundance of 380 bp fragment significantly decreased. The 4 major clinical recipes for Pi-deficiency recovered the activities of Pi-deficient rats, especially Si Junzi Tang and Buzhong Yiqi Tang at their medium dose (equivalent to the clinical dose). These results strongly support the rationale behind the current common use of these two recipes for Pi-deficiency[12,14,19]. However, it should be noted that the higher dose of these recipes did not show a better therapeutic effect on Pi-deficiency in the present study. A possible explanation of this phenomenon might be that the recipes have some anti-microorganism actions on Pi-deficiency. It was reported that Si Junzi Tang and some TCM recipes have certain modulating functions in intestinal flora[20-22].

The pathogenesis of Pi-deficiency induced by *Folium Sennae* or by *Radix* and *Rhizoma Rhei* is similar. *Folium Sennae, Radix* and *Rhizoma Rhei*, classified as "bitter-cold" in terms of taste and properties, can simulate the intestinal motility and secretion to induce diarrhea, which results in Pi-deficiency. However, the diarrhea-inducing action of *Folium Sennae* is weaker than that of *Radix* and *Rhizoma Rhei*. That is why the four recipes showed a better recovery profile for *Folium Sennae*-induced Pi-deficiency than that for *Radix* and *Rhizoma Rhei*-induced Pi-deficiency, in the present study.

In conclusion, Pi-deficiency, one of the most common digestive system diseases, is generally caused by the change in intestinal microflora. Although the underlying mechanism of action of TCM is not completely understood, it has been known that TCM has positive effects on some syndromes including Pi-deficiency. ERIC-PCR fingerprints can be used to screen changes in composition of bacterial communities associated with the development of intestinal disease, and to investigate the pharmacodynamic effect of TCM on intestinal microflora or intestinal diseases such as Pi-deficiency.

COMMENTS

Background

Pi-deficiency, a clinical syndrome in traditional Chinese medicine (TCM), is one of the most common digestive system diseases and generally considered to be associated with abnormalities of gastrointestinal microflora.

Research frontiers

Although the underlying mechanism of action of TCM is not completely understood, it has been known that TCM has positive effects on some syndromes including Pi-deficiency. It was reported that some TCM have certain modulating functions in intestinal flora.

Innovations and breakthroughs

In this study, the authors used the molecular markers in study of Pi-deficiency

syndrome, changes in intestinal microflora, and evaluation of the therapeutic effects of traditional Chinese drugs. This is the first study reporting the changes in intestinal microflora of Pi-deficient rats using the enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprint profiles.

Applications

ERIC-PCR fingerprints can be used to screen changes in the composition of bacterial communities associated with the development of intestinal disease, and to investigate the pharmacodynamic effect of TCM on intestinal microflora or intestinal diseases including Pi-deficiency.

Terminology

ERIC-PCR is a PCR-based technique in which DNA is isolated from a mixed sample and amplified using conserved ERIC primers targeting short repetitive sequences which are dispersed throughout various bacterial genomes.

Peer review

The authors identified the molecular markers of intestinal microflora by modified ERIC-PCR in rats with Pi-deficiency induced by administration of *Radix* and *Rhizoma Rhei*. In addition, data on the effect of several decoctions on P-deficiency induced by *Radix* and *Rhizoma Rhei* are interesting and seem reliable. The study is interesting and well-designed.

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ORIGINAL ARTICLES

FAT10 level in human gastric cancer and its relation with mutant p53 level, lymph node metastasis and TNM staging

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Abstract

AIM: To investigate the role of FAT10 and mutant p53 in the pathogenesis, severity and prognosis of gastric cancer.

METHODS: FAT10, mutant p53 mRNA and protein levels were measured by reverse transcription (RT)-PCR and immunohistochemistry in gastric cancer tissue (n = 62), tumor-adjacent tissue (n = 62) and normal gastric tissue (n = 62). Relation of FAT10 and mutant p53 expression with clinicopathological features and clinical outcomes of gastric cancer patients were analyzed.

RESULTS: The FAT10, mutant p53 mRNA and protein levels were significantly higher in gastric cancer than in its adjacent and normal tissue. The FAT10 and mutant p53 levels in gastric cancer tissue were significantly correlated with lymph node metastasis and tumor, nodes, metastasis (TNM) staging. Moreover, the high FAT10 level was associated with the overall survival rate of patients. Multivariate Cox-proportional hazards model analysis showed that mRNA and protein levels of FAT10 and mutant p53, lymph node metastasis, distant metastasis and TNM stage were the independent prognostic factors for gastric cancer.

CONCLUSION: FAT10 may be involved in gastric carcinogenesis, and is a potential marker for the prognosis of gastric cancer patients. FAT10 and mutant p53 may play a common role in the carcinogenesis of gastric cancer.

INTRODUCTION

FAT10, also known as diubiquitin, is a ubiquitin-like modifier (UBL) of the ubiquitin protein family, first discovered by Fan *et al*[1] in mapping HLA-F gene in 1996. It has been shown that FAT10 is expressed in mature B cells and dendritic cells[2]. It has been reported that FAT10 regulates cell-cycle and non-covalently binds to the human spindle assembly checkpoint protein (MAD2) that is responsible for the maintenance of spindle integrity during mitosis. Inhibition of MAD2 may lead to chromosomal instability, a common feature of tumorigenesis[3,4]. Lee *et al*[5] found that FAT10 is up-regulated in liver, uterine cervix, ovarian, rectal, pancreatic cancers and small intestinal adenocarcinoma, suggesting that FAT10 plays an important role in tumorigenesis.

P53 gene is located on the short arm of chromosome 17 and classified into wild *p53* and mutant *p53*. P53 protein depletion or gene mutation has been detected in over 50% of all cancers. Under the regulation of upstream signals such as DNA damage, proto-oncogene activity, spindle damage and hypoxia, p53 is activated and functions as a modifier in the processes of cell apoptosis, cell cycle arrest, and DNA repair[6,7]. It was reported that damaged DNA enters into S stage, changes cell hereditary characteristics, and finally induces tumorigenesis when p53 is deleted or mutated[8]. There is evidence that the p53 mutation rate is higher in gastric cancer with atrophic gastritis than in that without atrophic gastritis[9]. Rugge *et al*[10] demonstrated

that the *p53* gene mutation rate is 9.7% in patients (less than 40-year old) with gastric cancer of intestinal type, and lower than in old people (40%-60%) and that the p53 mutation rate is lower in young (6.8%) than in old people (10%-25%) with gastric cancer of diffuse type. Moreover, *Helicobacter pylori* (*H pylori*) infection is an important risk factor for gastric tumorigenesis, whereas patients with gastric cancer and *H pylori*-related cytotoxin-associated gene (CagA) are often accompanied with p53 mutation[11], suggesting that p53 gene mutation also plays an important role in gastric tumorigenesis.

Gastric cancer, one of the most common malignant tumors, is a leading cause of cancer-related death worldwide. The mortality of male and female patients with gastric cancer is on the top of the list in China[12]. Although various genetic and molecular alterations have been found in gastric cancer that underly the malignant transformation of gastric mucosa during the multi-step process of carcinogenesis, the detailed mechanism underlying the development of gastric cancer still remains uncertain. It has recently proposed that FAT10 is a downstream target of p53, and dysregulation of FAT10 expression in p53-defective cells can contribute to carcinogenesis[13]. Therefore, it would be of importance if the function of both FAT10 and p53 and their correlation are investigated in human beings. Furthermore, although many researches are available on the structure and function of FAT10, and its inducing factors, little is known about the role of FAT10 in gastric tumorigenesis and its relation with mutant p53 and other gastric cancer biomarkers. In the present study, we analyzed the expression of FAT10 and mutant p53 in gastric cancer tissue and its adjacent tissue and normal gastric mucosa tissue, in an attempt to discover the potential role of FAT10 in the development of gastric cancer.

MATERIALS AND METHODS

Gastric cancer specimens

In this study, gastric cancer tissue and its adjacent tissue (within 2 cm next to the margin of tumor tissue) and normal gastric tissue (more than 5-10 cm next to the margin of tumor tissue) were obtained from 62 gastric cancer patients who were underwent surgical resection in the First Affiliated Hospital of Medical College, Zhejiang University, from March 2003 to May 2004. None of the patients received any preoperative therapies such as chemotherapy or radiotherapy. The patients consisted of 38 males and 24 females, their age ranged 21- 86 years (mean age: 59.62 years). The tumor, nodes, metastasis (TNM) stage of gastric cancer referring to the p-TNM stage were promulgated by the International Union against Cancer (UICC) in 1997. This study was approved by the Hospital Review Board and written consent was obtained from each involved patient.

Immunohistochemistry

FAT10 and mutant p53 protein levels were routinely measured by immuno-histochemistry. Briefly, gastric cancer tissue and its adjacent tissue and normal gastric tissue were sequentially fixed with 10% formalin, embedded in paraffin and cut into 4-μm thick sections. The sections were deparaffinaged and endogenous peroxidase was blocked with H_2O_2. Antigen retrieval was performed by heating the sections in a 0.01 mol/L citrate buffer in a microwave oven. Nonspecific binding was blocked by incubating the sections with normal rabbit serum for 20 min. The sections were then incubated at 37℃ for 2 h with either polyclonal FAT10 antibody (Shanghai Jintai Biological Science and Technology Ltd, China) or monoclonal mutant P53 antibody (Beijing Zhongshan Biological Science and Technology Ltd, China). Controls without primary antibodies were also included. After washed three times with PBS, the sections were incubated with biotin-conjugated secondary antibody (Shanghai Jintai Biological Science and Technology Ltd, China) for 40 min at room temperature. Immunocomplexes were detected with 3, 3-diaminobenzidine (Fuzhou Maixin Biological Science and Technology Ltd, China) that acts as a chromogen and results in deposition of brown reaction products. Species with 0%, about 10%, about 50% of positively stained cells were scored as -, +, and ++, respectively.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted using Trizol reagent with one-step extraction method. In brief, 1 mL Trizol reagent was added to cultured cells or to approximately 100 mg tissue specimens, respectively, and total RNA was isolated following the manufacturer's instructions. RNA integrity was assessed by agarose gel electrophoresis when clear 18 S and 28 S strips appeared on the gel, whereas total RNA concentration was measured with a spectrophotometer ($A_{260/280}$ Ratios of 1.8-2.0) following its manufacturer's instructions. Total RNA (5 μL) was reversed into cDNA in a 24 μL reaction system at 42℃ for 60 min and at 70℃ for 10 min, sequentially. The mixture contained 5 μL 5-RT-buffer, 1 μL Oligo (dT), 2 μL 10 mmol/L dNTPs mix, 1 μL ribonuclease inhibitor (20 U/μL), 1 μL M-MMLV reverse transcriptase (200 U/μL), and 9 μL DEPC water. The final RT reaction solution was used in PCR (GeneAmp 2400 PCR System, Perkin Elmer Company, USA). The primers and lengths of PCR amplifications are listed in Table 1. DNA products were run in agarose gel at 80-100 V and then analyzed by BIO-RAD image acquisition with an analysis system, followed by semi-quantitative analysis with Quantity One software. The 295 bp, 478 bp, and 492 bp long fragments were detected under UV light. Gray scale scanning was performed on electrophoresis strips using an image acquisition and analysis system.

Statistical analysis

Immunohistochemical expression was defined as positive when moderate to strong nuclear staining was observed in more than 10% cells. χ^2 test was used to determine

Table 1 Primers used in this study

Designation	Sequences	PCR products (bp)
β-actin		
Upstream primer	5'-TCACCCACACCGT-GCCCATCTACGA-3'	295
Downstream primer	5'-CAGCGGAACCGCT-CATTGCCAACGG-3'	
FAT10		
Upstream primer	5'-AATGCTTCCTGCCTCT-GTGT-3'	478
Downstream primer	5'-GCCGTAATCTGCCAT-CATCT-3'	
Mutant p53		
Upstream primer	5'-CCTATGGAAAC-TACTTCCTGAAAACAA-3'	492
Downstream primer	5'-ACAGCATCAAAT-CATCCATTGC-3′	

the difference in FAT10 and p53 protein levels between different groups and the correlation between positive FAT10 ratio and clinicopathological parameters of patients. RT-PCR semi-quantitative results were expressed as mean ± SD. Student's *t*-test was employed to analyze differences in mRNA levels. Relation between FAT10 and p53 was statistically analyzed using Spearman's rank correlation. Overall survival rate was calculated by Kaplan-Meier curves. Cox proportional hazards model was used to examine the effect of potential prognostic variables on survival. Statistical analysis was performed with SPSS software version 12.0. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of FAT10 and mutant p53 protein in different gastric tissues by immunohistochemistry

FAT10 protein was mainly detected in nuclei of malignant and benign cells which were stained brown-yellow (Figure 1A). Positive staining of FAT10 cells was found in tumor tissue and its adjacent tissue and normal tissue samples from 32 (51.61%), 8 (12.90%) and 4 (6.45%) of the 62 cases. The FAT10 level was significantly different in gastric cancer tissue, and its adjacent tissue and normal tissue ($\chi^2 = 40.96$, $P < 0.01$). The rate of positive FAT10 cells in gastric cancer tissue was significantly higher than that in its adjacent tissue and normal tissue ($P < 0.01$) but was not significantly different between adjacent tissue and normal tissue (Table 2). Mutant P53 was expressed mainly in nuclei and cytoplasm (Figure 1B). Positive immunostaining of mutant fp53 was noted in 45.16% (28/62) of tumor tissue samples, 14.51% (9/62) of adjacent tissue samples and 9.63% (6/62) of normal tissue samples ($\chi^2 = 25.83$, $P < 0.01$). The positive rate of mutant p53 expression in tumor tissue was significantly higher than that in adjacent tissue and normal tissue ($P < 0.01$, Table 2).

Detection of FAT10 and mutant p53 mRNA in different gastric tissues by RT-PCR

The levels of FAT10 and mutant p53-mRNA in tumor tissue and non-tumor tissue were measured. RT-PCR analysis revealed that the relative FAT10-mRNA expression in gastric cancer tissue was significantly higher than that in its adjacent tissue ($t = 3.12$, $P < 0.01$) and normal tissue ($t = 4.64$, $P < 0.01$), whereas no significant difference was detected between tumor-adjacent and normal tissues ($t = 1.03$, Figure 2). Mutant P53-mRNA expression was significantly higher in gastric cancer tissue than in its adjacent tissue ($t = 6.79$, $P < 0.01$) and normal tissue ($t = 5.51$, $P < 0.01$). The difference in mutant p53-mRNA expression between adjacent and normal tissue was not statistically significant ($t = 1.22$, Figure 2).

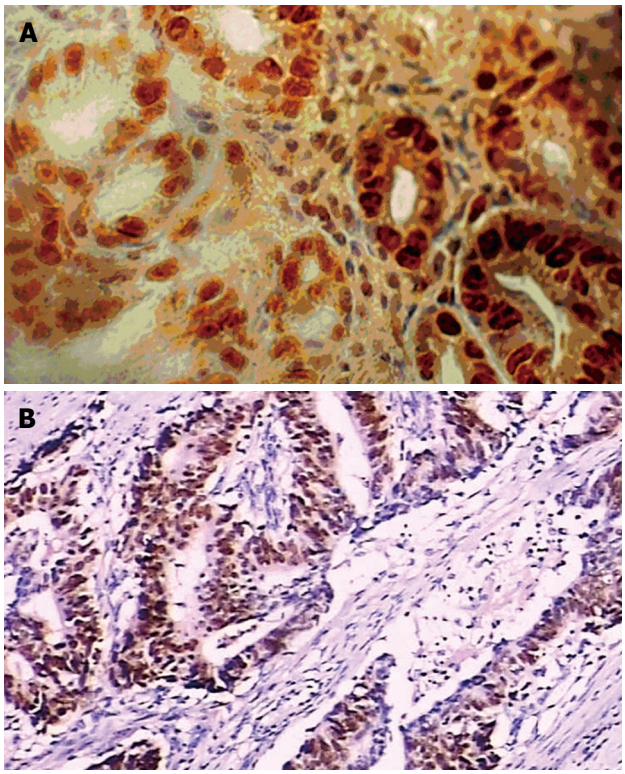

Figure 1 Expression of FAT10 (A) and mutant p53 (B) in gastric cancer tissue (× 400).

Table 2 FAT10 and mutant p53 protein expression in gastric cancer tissue and its adjacent tissue and normal tissue *n* (%)

Tissue	FAT10		Mutant p53	
	Negative	Positive	Negative	Positive
Gastric tissue	30	32 (51.61)	34	28 (45.16)
Tumor-adjacent tissue	54	8 (12.90)	53	9 (14.51)
Normal gastric tissue	58	4 (6.45)	56	6 (9.68)

Relation between FAT10 protein and mRNA levels and clinicopathological features of gastric cancer

To test the potential value of FAT10 as a gastric cancer biomarker, we performed χ^2 test to evaluate the correlation of FAT10 expression with clinicopathological features of gastric cancer (Table 3). The positive rate of FAT10 expression in gastric cancer with regional lymph node metastasis was significantly higher than that without

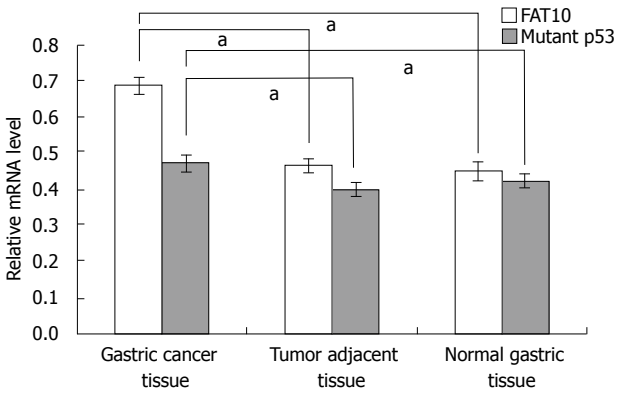

Figure 2 Relative mRNA levels of FAT10 and mutant p53 in different tissues. The column represents the relative gray values of FAT10 and mutant p53 mRNA by normalizing the gray value of β-actin. $^aP < 0.05$ (for FAT10: 0.689 ± 0.023 in gastric cancer tissue, 0.463 ± 0.019 in tumor adjacent tissue, 0.451 ± 0.028 in normal gastric tissue; for mutant p53: 0.471 ± 0.021 in gastric cancer tissue, 0.398 ± 0.017 in tumor adjacent tissue, 0.421 ± 0.019 in normal gastric tissue).

Table 3 Relation between FAT10 expression and clinicopathologic factors for gastric cancer

Clinic pathologic factors	Samples	FAT10 positive samples (%)	χ^2	*P*
Age (yr)				
< 50	19	7 (36.84)	2.39	> 0.05
⩾ 50	43	25 (58.14)		
Gender				
Male	38	19 (50.00)	0.1	> 0.05
Female	24	13 (54.16)		
Tumor size (cm)				
< 5	33	16 (48.48)	0.28	> 0.05
⩾ 5	29	16 (55.17)		
Location				
Antrum	31	15 (48.39)	2.81	> 0.05
Angle	5	2 (40.00)		
Body	14	9 (64.29)		
Fundus	4	3 (75.00)		
Cardia	8	3 (37.50)		
Progression degree				
Early stage	11	6 (54.54)	0.05	> 0.05
Progressive stage	51	26 (50.98)		
Differentiation degree				
High, middle	15	7 (46.67)	0.19	> 0.05
Low, none	47	25 (53.19)		
Lymph metastasis				
Positive	39	25 (64.10)	6.57	< 0.05
Negative	23	7 (30.43)		
Distant metastasis				
Positive	15	11 (73.33)	3.74	> 0.05
Negative	47	21 (44.68)		
TNM Staging				
Ⅰ + Ⅱ	21	7 (33.33)	4.25	< 0.05
Ⅲ + Ⅳ	41	25 (60.98)		

regional lymph node metastasis ($P < 0.05$). Furthermore, high FAT10 expression levels were associated with advanced TNM staging (Ⅲ + Ⅳ/ Ⅰ + Ⅱ) ($P < 0.05$). However, there was no significant difference in FAT10 expression, age and gender of patients, tumor size, location, histological grade, and distant metastasis. The expression of FAT10-mRNA was correlated with lymph node status and TNM stage (Ⅲ + Ⅳ/ Ⅰ + Ⅱ)

Table 4 Relation between FAT10-mRNA level and clinicopathological factors for gastric cancer

Clinicopathological factors	Samples (*n*)	Positive samples	Strap gray value (mean ± SD)	*t*-value	*P*
Age (yr)					
< 50	19	10	0.583 ± 0.036	1.12	> 0.05
⩾ 50	43	26	0.611 ± 0.026		
Sex					
Male	38	21	0.622 ± 0.013	1.36	> 0.05
Female	24	15	0.574 ± 0.024		
Tumor size (cm)					
< 5 cm	33	19	0.594 ± 0.022	0.65	> 0.05
⩾ 5 cm	29	17	0.590 ± 0.018		
Location					
Tantrum	31	17	0.537 ± 0.019	1.54	> 0.05
Angle	5	3	0.672 ± 0.037		
Body	14	9	0.594 ± 0.021		
Fundus	4	4	0.611 ± 0.025		
Cardia	8	3	0.529 ± 0.032		
Progression degree					
Early	11	8	0.623 ± 0.023	1.15	> 0.05
Advanced	51	28	0.597 ± 0.020		
Differentiation degree					
Well-middle	15	9	0.564 ± 0.031	1.39	> 0.05
Low-non	47	27	0.595 ± 0.019		
Lymph node metastasis					
Positive	39	27	0.656 ± 0.016	3.37	< 0.01
Negative	23	9	0.531 ± 0.026		
Distant metastasis					
Positive	15	12	0.623 ± 0.033	1.74	> 0.05
Negative	47	24	0.598 ± 0.017		
TNM stage					
Ⅰ + Ⅱ	21	8	0.667 ± 0.023	2.25	< 0.05
Ⅲ + Ⅳ	41	28	0.558 ± 0.015		

Table 5 Correlation between FAT10 and mutant p53 expressions in gastric cancer tissue

p53	Samples	FAT10 expression	
		Positive	Negative
Positive	28	23	5
Negative	34	9	25
Total	62	32	30

($P < 0.05$), but not with age and sex of patients, tumor size, location, histological grade, and distant metastasis, which was similar to the immunohistochemical results (Table 4).

Correlation between FAT10 and mutant p53 in gastric cancer tissue

The expression rate of FAT10 was 82.14% (23/28) and 26.47% (9/34) in positive and negative mutant p53 tumor tissues, respectively (Table 5). The correlation between FAT10 and mutant p53 was analyzed by Spearman rank correlation. The expression of FAT10 was positively correlated with mutant p53 in gastric cancer ($r = 0.865$, $P < 0.05$). Furthermore, Spearman rank correlation analysis showed that FAT10-mRNA was significantly correlated with mutant p53-mRNA ($r = 0.548$, $P < 0.05$).

Predictive role of FAT10 in gastric cancer

To investigate the impact of FAT10 over-expression

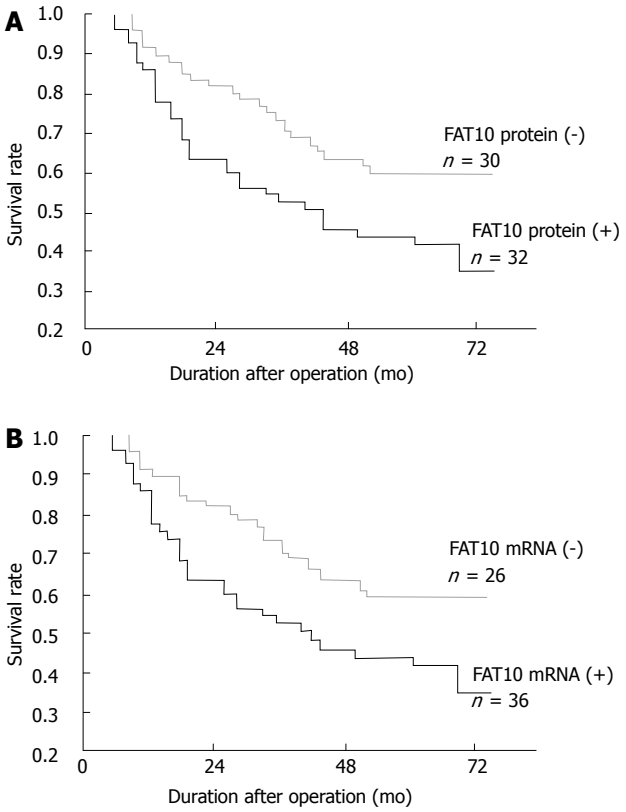

Figure 3 Effect of FAT10 protein (A) and mRNA (B) on the survival rate of gastric cancer patients (Kaplan-Meier survival curve).

Table 6 Cox regression model analysis results of the prognostic factors for gastric cancer

Harzard factors	B	S χ	Wald	υ	Sig.	Exp (B)
Age	0.134	0.144	0.853	1	0.533	1.012
Tumor size	0.243	0.246	0.804	1	0.368	0.763
Location	0.072	0.055	1.846	1	0.173	0.954
Progression	0.144	0.267	0.26	1	0.615	1.148
Differentiation degree	0.544	0.23	2.613	1	0.118	1.223
Lymph node metastasis	0.734	0.384	4.985	1	0.027	1.886
Distant metastasis	0.764	0.326	6.785	1	0.001	1.806
TNM stage	0.698	0.285	7.205	1	0.007	1.745
FAT10 protein	0.661	0.228	8.448	1	0.004	1.516
FAT10 mRNA	0.793	0.245	7.658	1	0.003	1.854
p53 protein	0.669	0.239	6.479	1	0.006	1.611
p53 mRNA	0.758	0.346	6.857	1	0.004	1.521

on the clinical outcome of patients, univariate survival probability curves were plotted with respect to the immunohistochemical and RT-PCR results. Except for 4 patients (2 with positive FAT10 protein and FAT10-mRNA, and 2 with negative FAT10 protein and FAT10-mRNA), the other patients were followed up for 48-72 mo. We found that high FAT10 protein and mRNA levels in gastric cancer showed a tendency towards unfavorable prognosis regarding the overall survival rate as shown by Kaplan-Meier analysis ($P < 0.05$, Figure 3). In the Cox regression model, multivariate survival analyses showed that FAT10 protein and mRNA, as well as lymph node metastasis, distant metastasis, TNM stage, and mutant p53 mRNA protein, were the independent adverse prognostic factors for the overall survival rate (Table 6).

DISCUSSION

FAT10 is a member of the ubiquitin-like modifier family of proteins. Over-expression of the FAT10 gene has been observed in several epithelial cancers and high FAT10 expression can increase chromosome instability by reducing kinetochore localization of MAD2 during the prometaphase stage of cell-cycle[14]. In the present study, we measured the FAT10 protein and mRNA levels in gastric cancer tissue, its adjacent tissue and normal tissue from 62 patients. The immunohistochemical analysis suggested that FAT10 protein was mainly expressed in cell nuclei, indicating that FAT10 may participate in cell cycle regulation. The positive expression rate of FAT10 protein and mRNA in gastric cancer tissue was significantly higher than that in its adjacent tissue and normal tissue, suggesting that FAT10 may play an important role in the process of gastric carcinogenesis

Recent researches have shown that the expression of mutant p53 and FAT10 mRNAs is increased in cancer cell line[15,16]. However, the correlation between mutant p53 and FAT10 has not been analyzed in human gastric cancer. In this study, FAT10 and mutant p53 protein/mRNA were over-expressed in gastric cancer tissue whereas high FAT10 and mutant p53 expression levels in tumor tissue were positively correlated. It was reported that p53 negatively regulates the expression of FAT10 and p53 depletion, thus contributing to tumorigenesis by uncontrolled up-regulation of FAT10[13], suggesting that mutant p53 may also activate the FAT10 gene and promote gastric tumorigenesis due to the loss of its anti-carcinoma effect. Moreover, proinflammatory cytokines up-regulate FAT10 in liver and colon cancer, indicating that they play a potential role in activating FAT10 in gastric tumor and merit further investigation[16]. Although p53 binds to the 5' half consensus sequence of p53-binding site at the FAT10 promoter[13], the exact p53-binding site is still unclear, thus further study is needed.

No report is available at present on the correlation between FAT10 protein expression and clinicopathological characteristics of gastric cancer patients. In the present study, FAT10 protein and mRNA levels were closely correlated with lymph node metastasis and TNM stage (Ⅲ + Ⅳ/ Ⅰ + Ⅱ) ($P < 0.05$), indicating that FAT10 can promote tumor invasion and metastasis, and may be a candidate prognostic factor for lymph node metastasis and tumor progression. Large scale studies are needed to further confirm our findings.

Tumor metastasis has become one of the most challenging problems in tumor therapy. Many efforts have been made to predict gastric cancer behaviors, but specific predictive markers for metastasis and recurrence are still lacking[17]. In our study, the patients with a high FAT10 expression level showed a tendency towards unfavorable prognosis. Since FAT10 and mutant p53 protein and mRNA, lymph node

metastasis, distant metastasis, and TNM stage are the independent prognostic factors for poor patient survival, determination of FAT10 status may be an important step in formulating right therapeutic strategies. Moreover, FAT10 may be related with other predictive biomarkers of tumor metastasis, such as CD44v6, nm23, MTA1 and MMPs.

In conclusion, FAT10 is over-expressed in gastric cancer tissue, and positively correlated with mutant p53 expression, lymph node metastasis and tumor progression, and can promote tumor invasion. FAT10 is of prognostic value for human gastric cancer and is a potential target for cancer biotherapy.

COMMENTS

Background

FAT10 belongs to the ubiquitin-like modifiers of ubiquitin protein family, first discovered in mapping HLA-F gene in 1996, and is expressed in mature B cells and dendritic cells. It has been reported that FAT10 can regulate cell-cycle and may play an important role in tumorigenesis. *P53* gene, located on the short arm of chromosome 17, can be divided into wild *p53* and mutant *p53*. P53 protein depletion or gene mutation has been detected in over 50% of all cancers, suggesting that P53 may play an important role in gastric tumorigenesis. Gastric cancer, one of the most common malignant tumors, is a leading cause of cancer-related death worldwide, and is on the top of the list in China. The detailed mechanism underlying the development of gastric cancer still remains uncertain.

Research frontiers

It has been recently found that FAT10 can non-covalently bind to the human spindle assembly checkpoint protein (MAD2) that is responsible for the maintenance of spindle integrity during mitosis. Inhibition of MAD2 may lead to chromosomal instability, a common feature of tumorigenesis. It has been shown that damaged DNA enters into S stage, changes cell hereditary characteristics, and finally induces tumorigenesis when p53 is deleted or mutated. Moreover, there is evidence that the p53 mutation rate is higher in gastric cancer with atrophic gastritis than in gastric cancer without atrophic gastritis. FAT10 is a downstream target of p53, and down-regulation of FAT10 expression in p53-defective cells contributes to carcinogenesis.

Innovations and breakthroughs

In this study, the FAT10 and mutant p53 mRNA and protein levels were significantly higher in gastric cancer tissue than in its adjacent tissue and normal tissue. The FAT10 and mutant p53 levels in gastric cancer tissue were significantly correlated with lymph node metastasis and TNM staging. Moreover, mRNA and protein levels of FAT10 and mutant p53, lymph node metastasis, distant metastasis, and TNM stage were found to be independent prognostic factors for patients with gastric cancer.

Applications

FAT10 may be a potential marker of gastric cancer prognosis, which needs to be further verified. FAT10 is positively correlated with mutant p53, indicating that it may play a role in carcinogenesis and becomes a novel therapeutic target of gastric cancer.

Terminology

FAT10: a protein belonging to ubiquitin-like modifier (UBL) of ubiquitin protein family, is mainly expressed in mature B cells and dendritic cells and regulates cell-cycle and chromosomal instability, thus playing an important role in tumorigenesis.

Peer review

This study describes the increased FAT10 and mutant p53 mRNA and protein levels in gastric cancer and the correlation between FAT10 and mutant p53. Furthermore, the authors also found that mRNA and protein levels of FAT10 were independent prognostic factors for patients with gastric cancer. These results are innovative, showing that FAT10 may be involved in gastric carcinogenesis and in gastric cancer prognosis. However, further study is needed to further verify their findings.

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ORIGINAL ARTICLES

Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gen-difluoromethylenechrysin

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Abstract

AIM: To investigate the effect of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) on apoptosis of human liver carcinoma HepG2 cell line and the molecular mechanisms involved.

METHODS: HepG2 cells and L-02 cells were cultured *in vitro* and the inhibitory effect of ADFMChR on their proliferation was measured by MTT assay. The apoptosis of HepG2 cells was determined by flow cytometry (FCM) using propidium iodide (PI) fluorescence staining. DNA ladder bands were observed by DNA agarose gel electrophoresis. The influence of ADFMChR on the proxisome proliferator-activated receptor γ (PPARγ), NF-κB, Bcl-2 and Bax protein expression of HepG2 cells were analyzed by Western blotting.

RESULTS: MTT assay showed that ADFMChR significantly inhibited proliferation of HepG2 cells in a dose-dependent manner, with little effect on growth of L-02 cells, and when IC_{50} was measured as 8.45 μmol/L and 191.55 μmol/L respectively, the potency of ADFMChR to HepG2 cells, was found to be similar to 5-fluorouracil (5-FU, IC_{50} was 9.27 μmol/L). The selective index of ADFMChR cytotoxicity to HepG2 cells was 22.67 (191.55/8.45), higher than 5-FU (SI was 7.05 (65.37/9.27). FCM with PI staining demonstrated that the apoptosis rates of HepG2 cells treated with 3.0, 10.0 and 30.0 μmol/L ADFMChR for 48 h were 5.79%, 9.29% and 37.8%, respectively, and were significantly higher when treated with 30.0 μmol/L ADFMChR than when treated with 30.0 μmol/L ChR (16.0%) ($P < 0.05$) and were similar to those obtained with 30.0 μmol/L 5-FU (41.0%). DNA agarose gel electrophoresis showed that treatment of HepG2 cells with 10.0 μmol/L ADFMChR for 48 h and 72 h resulted in typical DNA ladders which could be reversed by 10.00 μmol/L GW9662, a blocker of PPARγ. Western blotting analysis revealed that after 24 h of treatment with 3.0, 10.0, 30.0 μmol/L ADFMChR, PPARγ and Bax protein expression in HepG2 cells increased but Bcl-2 and NF-κB expression decreased; however, pre-incubation with 10.0 μmol/L GW9662 could efficiently antagonize and weaken the regulatory effect of 3.0, 30.0 μmol/L ADFMChR on PPARγ and NF-κB protein expression in HepG2 cells.

CONCLUSION: ADFMChR induces apoptosis of HepG2 cell lines by activating PPARγ, inhibiting protein expression of Bcl-2 and NF-κB, and increasing Bax expression.

Key words: Liver neoplasm; Chrysin; 5-allyl-7-gen-difluoromethylenechrysin; Apoptosis; Proxisome proliferator-activated receptor γ

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Tan XW, Xia H, Xu JH, Cao JG. Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gen-difluoromethylenechrysin. *World J Gastroenterol* 2009; 15(18): 2234-2239 Available from: URL: http://www.wjgnet.com/1007-9327/15/2234.asp DOI: http://dx.doi.org/10.3748/wjg.15.2234

INTRODUCTION

Human liver carcinoma is the fifth most common cancer in the world and is responsible for > 600 000 deaths

annually[1]. The majority of patients with hepatocellular carcinoma die within 1 year after the diagnosis. At present, the treatment of hepatocellular carcinoma mainly includes surgery and chemotherapy, but the curative effects of the existing chemotherapeutic drugs are not good enough and they have numerous side effects. Therefore, searching for highly efficient antitumor drugs remains a hot research area.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily; a ligand-dependent transcription factor that plays an important role in lipid and glucose metabolism[2,3]. In recent years, over-expression of PPARγ has been found in a variety of tumor cells and PPARγ agonists can induce apoptosis[4,5]. It has been reported that chrysin (ChR) and its derivatives activate PPARγ to inhibit COX-2 and iNOS activity through various pathways distinguished from thiazolidones[6].

Chrysin (5,7-dihydroxy flavone, ChR) is a kind of flavonoid with pharmacological activities and is widely distributed in the plant kingdom. It has been demonstrated that ChR can markedly inhibit the growth of human thyroid cancer cells[7], and has an effect on the inhibition of proliferation and induction of apoptosis in human myeloid leukemia cells as well[8,9]. Comte *et al*[10] reported that, through alkylation, the hydrophobicity of ChR is increased, its KD value decreased, and its binding affinity towards P-glucoprotein (P-gp) enhanced. We confirmed that a series of B-ring trifluoromethylated derivatives of ChR markedly inhibited the growth of HT-29 and SGC-7901 cell lines[11] and that 5, 7-dihydroxy-8-nitrochrysin (NOChR) had an inhibitory effect on subcutaneously transplanted primary Lewis lung carcinoma in mouse and its spontaneous metastasis in a dose-dependent manner[12]. Our previous study showed that the suppressive effect of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) on proliferation of the CoC1 cell line was stronger than that of ChR[13]. However, whether ADFMChR has antitumor effects on human liver carcinoma is unknown.

In this study, we aimed to investigate whether ADFMChR induces apoptosis of HepG2 cell line by activation of PPARγ and whether NF-κB, Bcl-2 and Bax are involved in this mechanism, thereby providing a new opportunity for research with regard to the pharmaceutical prevention and cure of human liver cancer.

MATERIALS AND METHODS

Cell lines and cell culture

HepG2 cells and L-02 cells were purchased from the China Center for Type Culture Collection (CCTCC) and were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc) at 37℃ in a 5% CO_2 incubator.

Medicines and chemical reagents

ADFMChR was synthesized in the Medical College, Hunan Normal University as previously described[14]; with a molecular weight of 344 ku, characteristic yellow crystals and purity of 99.0%, its molecular formula is $C_{19}H_{14}O_4F_2$. ADFMChR was dissolved in dimethyl sulfoxide (DMSO), diluted with phosphate buffer solution (PBS), and finally prepared as 2 mmol/L storage solution after filtration sterilization. RPMI-1640, ChR, MTT and DMSO were purchased from Sigma Company. 5-fluorouracil (5-FU) was from Jinghua Pharmaceutical Corporation Ltd, Nantong. Ladder Apoptotic DNA Ladder Detection Kit was purchased from Bodataike Company, Beijing. Mouse anti-human Bcl-2 monoclonal antibody, mouse anti-human NF-κB monoclonal antibody, mouse anti-human Bax monoclonal antibody and rabbit anti-human PPARγ polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc (U.S.A).

MTT assay

HepG2 cells or L-02 cells were seeded in a 96-well plate at a density of 1.0×10^4 cells/well as previously described[15]. Drugs of different concentrations were added to each well and cultured for 48 h, followed by incubation with 5 mg/L MTT for 4 h. The supernatant was removed after centrifugation. Finally, 100 μL of DMSO was added and absorbance at 490 nm wavelength (A_{490}) was measured by means of Enzyme-labeling instrument (EX-800 type). Relative cell proliferation inhibition rate (IR) = (1 - average A_{490} of the experimental group/average A_{490} of the control group) × 100%.

Flow cytometry (FCM) with propidium iodide (PI) staining

HepG2 cells were treated with serum-free medium for 24 h, followed by treatment with media containing 3.0, 10.0, 30.0 μmol/L ADFMChR, 30.0 μmol/L ChR and 30.0 μmol/L 5-FU for 48 h, respectively. Cells were collected and prepared as a single cell suspension by mechanical blowing with PBS, washed with cold PBS twice, fixed with 700 mL/L alcohol at 4℃ for 24 h, stained with PI and cell apoptosis was detected using FCM (American BD Company, FACS420).

DNA agarose gel electrophoresis

As previously described[16], cells were cultured with 10.0 μmol/L ADFMChR and 10.0 μmol/L ADFMChR plus 10.0 μmol/L GW9662, a PPARγ antagonist, for 0, 24, 48 and 72 h, respectively. Cells were washed twice with PBS and DNA was extracted with an Apoptotic DNA Ladder Detection Kit according to the manufacturer's instructions. The extracted DNA was kept at 4℃ overnight. Then 8.5 μL of DNA sample was mixed with 1.5 μL of 6 × Buffer solution, electrophoresed on 20.0 g/L agarose gel containing ethidium bromide at 40 V, and observed through DBT-08 gel image analysis system.

Western blotting analysis

As previously described[17], cells were treated with 3.0, 10.0, 30.0 μmol/L ADFMChR and 30.0 μmol/L ChR for 24 h, respectively. Cells were collected, washed three times with PBS, lysed in cell lysis buffer containing 0.1 mol/L NaCl, 0.01 mol/L Tris-Cl (pH 7.6), 0.001 mol/L

EDTA (pH 8.0), 1 μg/mL Aprotinin, 100 μg/mL PMSF, and then centrifuged at 13000 × *g* for 10 min at 4℃. The extracted protein sample (25 μg total protein/lane) was added in the same volume of sample buffer and subjected to denaturation at 100℃ for 10 min, then electrophoresed on 100 g/L or 60 g/L SDS-PAGE at 100 mA for 3 h, and finally transferred onto PVDF membrane. The PVDF membrane was treated with TBST containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the primary antibodies PPARγ, NF-κB, Bcl-2 and Bax (1:500 dilution), respectively, at 37℃ for 2 h or at 4℃ overnight. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. The membrane was then washed three times for 15 min each with TBST. Fluorescence was visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). The results were analyzed with Image analyzer and the product of area and optical density was expressed as integral absorbance (IA).

Statistical analysis

Experimental data in each group were presented as mean ± SD. Analysis of variance was performed with SPSS software for windows 15.0 by using one way ANOVA and pairwise comparison with Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Determination of proliferation of HepG2 and L-02 cell lines by MTT assay

MTT assay showed that ADFMChR markedly inhibited proliferation of HepG2 cells in a dose-dependent manner (Figure 1), with little effect on growth of L-02 cells, and when IC_{50} were measured as 8.45 μmol/L and 191.55 μmol/L, respectively, the potency of ADFMChR to HepG2 cells was found to be similar to 5-fluorouracil (5-FU, IC_{50} was 9.27 μmol/L). The selective index of ADFMChR cytotoxicity to HepG2 cells was 22.67 (191.55/8.45), higher than 5-FU (SI was 7.05 (65.37/9.27).

Analysis of the effect of ADFMChR on apoptosis of HepG2 cell lines by FCM with PI staining

FCM with PI staining demonstrated that the apoptosis rates of HepG2 cells treated with 3.0, 10.0 and 30.0 μmol/L ADFMChR for 48 h were 5.79%, 9.29% and 37.8%, respectively, and were significantly higher when treated with 30.0 μmol/L ADFMChR than when treated with 30.0 μmol/L ChR (16.0%) ($P < 0.05$) and were similar to those obtained with 30.0 μmol/L 5-FU (41.0%) (Figure 2).

Detection of ADFMChR-induced apoptosis of HepG2 cells by agarose gel electrophoresis

DNA agarose gel electrophoresis showed that treatment of HepG2 cells with 10.0 μmol/L ADFMChR for 48 h and 72 h resulted in typical DNA ladders, which could be eliminated or attenuated by treating with 10.0 μmol/L ADFMChR plus 10.0 μmol/L GW9662 for 48 h and 72 h (Figure 3).

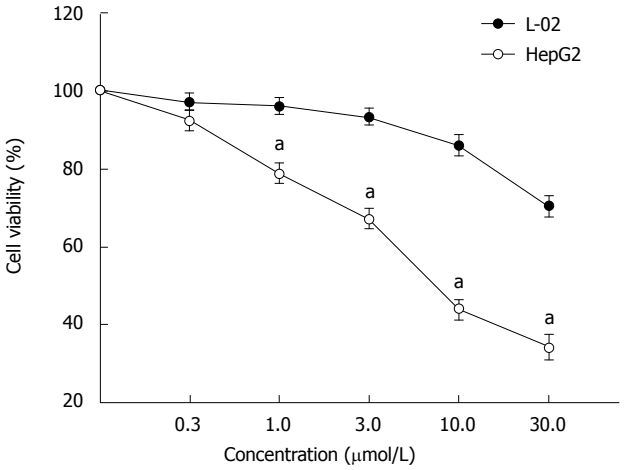

Figure 1 ADFMChR selectively inhibited proliferation of HepG2 cells. $^aP < 0.05$ *vs* treatment with ADFMChR in the same concentration to L-02 cells (mean ± SD, $n = 9$).

Analysis of the effect of ADFMChR on PPARγ, NF-κB, Bax and Bcl-2 protein expression of HepG2 cell line

Western blotting analysis showed that the relative densities of PPARγ, NF-κB, Bcl-2 and Bax protein bands of HepG2 cells treated with 3.0, 10.0, 30.0 μmol/L ADFMChR for 24 h were 109.3%, 126.4%, 147.7% and 92.9%, 89.0%, 72.4% and 94.1%, 85.5%, 77.3% and 106.8%, 116.3%, 125.7% of the HepG2 cells not treated with ADFMChR, respectively ($P < 0.05$) (Figure 4). This indicates that ADFMChR can increase the PPARγ and Bax protein expression and decrease NF-κB and Bcl-2 protein expression.

Effect of GW9662 on regulation of PPARγ and NF-κB protein expression by ADFMChR

Western blotting analysis demonstrated that when HepG2 cells were pre-incubated with 10.0 μmol/L GW9662, a blocker of PPARγ, for 30 min, the effects of 3.0, 30.0 μmol/L ADFMChR on PPARγ protein expression and NF-κB protein expression were antagonized or weakened (Figure 5), suggesting that the effects of ADFMChR on up-regulation of PPARγ protein expression and down-regulation of NF-κB protein expression were associated with the activation of PPARγ.

DISCUSSION

Tumorigenesis and tumor progression are strongly associated with abnormal apoptosis. A number of antitumor drugs exert their therapeutic effects by inducing or promoting apoptosis. Enhancing the antitumor effect of existing anticancer drugs, but not to increase its toxicity, is the aim of current anticancer research. There is evidence to support the concept that luteolin, apigenin and chrysin have great potential to be developed into novel cancer preventative agents[18].

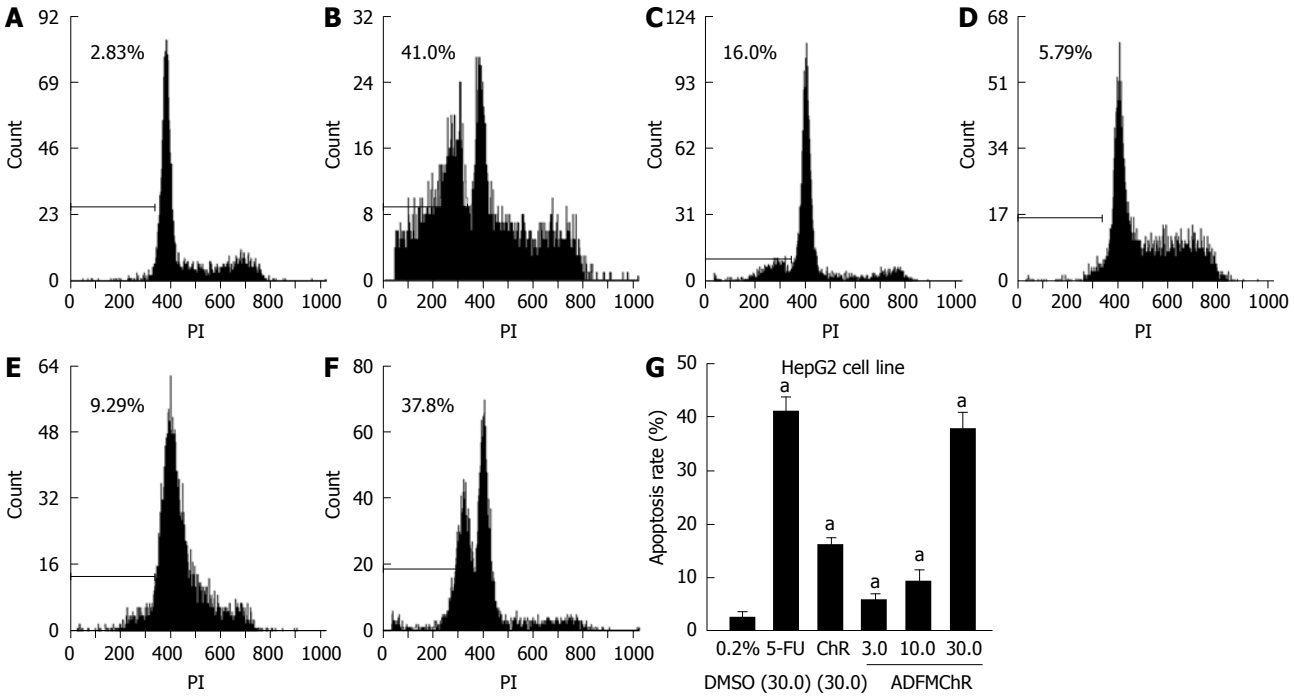

Figure 2 Induction of apoptosis of HepG2 cells by ADFMChR. A: Treated with 0.2% DMSO; B: Treated with 30.0 μmol/L 5-FU; C: Treated with 30.0 μmol/L ChR; D: Treated with 3.0 μmol/L ADFMChR; E: Treated with 10.0 μmol/L ADFMChR; F: Treated with 30.0 μmol/L ADFMChR; G: Quantification of induction of apoptosis analysis of HepG2 cells. $^aP < 0.05$ *vs* treatment with DMSO (mean ± SD, $n = 3$).

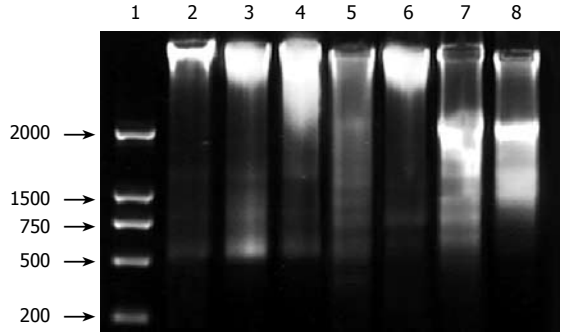

Figure 3 DNA ladder assay showing ADFMChR-induced apoptosis of HepG2 cells. Lane 1: DNA marker; lane 2: Control; lane 3: 10.0 μmol/L ADFMChR (24 h); lane 4: 10.0 μmol/L ADFMChR + GW9662 (24 h); lane 5: 10.0 μmol/L ADFMChR (48 h); lane 6: 10.0 μmol/L ADFMChR + GW9662 (48 h); lane 7: 10.0 μmol/L ADFMChR (72 h); lane 8: 10.0 μmol/L ADFMChR + GW9662 (72 h).

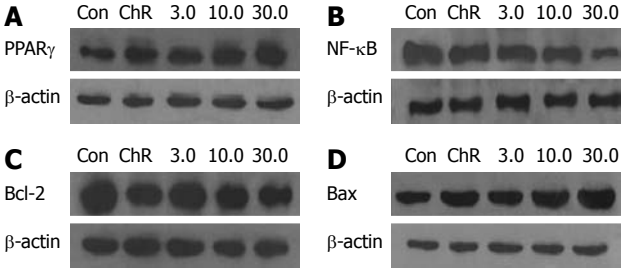

Figure 4 Western blotting analysis showing regulation of PPARγ (A), NF-κB (B), Bcl-2 (C) and Bax (D) protein expression in HepG2 cells by ADFMChR. (mean ± SD, $n = 3$).

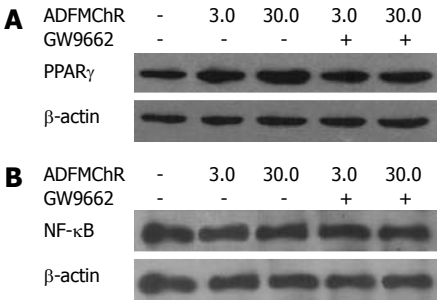

Figure 5 PPARγ antagonist GW9662 blocked the effects of ADFMChR on PPARγ and NF-κB protein expression in HepG2 cells. A: PPARγ; B: NF-κB. HepG2 cells were pretreated with 10.0 μmol/L GW9662 for 30 min, then exposed to 3.0, 30.0 μmol/L ADFMChR for 24 h, respectively (mean ± SD, $n = 3$).

Our previously research showed that ADFMChR potently inhibited the proliferation of ovarian cancer CoC1 cells in a dose-dependent manner[19], and could induce apoptosis of SMMC-7721 cells *in vitro*, with its mechanism possibly associated with G1 phase cell cycle arrest[20]. Li *et al*[19] and Xu *et al*[21] found that the ability of ADFMChR to induce induction of apoptosis in CoC1 cells may be mediated by activation of PPARγ, sequentially accompanied by reducing NF-κB and Bcl-2 levels and increasing Bax expression. Our experiment was to investigate the apoptosis of human liver carcinoma HepG2 cell line induced by ADFMChR and to provide experimental evidence for its application as an antitumor drug.

Apoptosis usually results in typical morphological and biochemical characteristics, including condensed chromatin in cells, appearance of apoptotic bodies, presence of hypodiploid peak in FCM analysis and DNA ladder bands on agarose electrophoresis[22,23]. In

this study, treatment of HepG2 cells with ADFMChR resulted in formation of DNA ladder bands and the appearance of marked hypodiploid peak. Thus, this experiment suggested that ADFMChR can induce apoptosis of human liver carcinoma HepG2 cell line *in vitro*.

PPARγ is a kind of ligand-activated nuclear transcription factor belonging to a nuclear receptor superfamily and has been implicated in metabolic diseases and is associated with cell proliferation, differentiation and apoptosis[24]. NF-κB inhibits apoptosis, promotes cell survival and reduces the expression of Bcl-2[25]. Chen *et al*[26] confirmed that PPARγ ligands may markedly inhibit NF-κB expression and reduce Bcl-2 expression leading to inhibited cell growth and induction of apoptosis of colonic cancer HT-29 cell line by activation of PPARγ. Liang *et al*[6] have recently shown that ChR is activated in different ways with thiazolidinones, and PPARγ inhibits activation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. 8-bromo-7-methoxychrysin (BrMChR) or 5,7-dihydroxy-8-nitrochrysin (NOChR) induce apoptosis of SGC2-7901 cell line by activating PPARγ[27,28]. In order to find out whether ADFMChR decreases NF-κB and Bcl-2 protein expression to induce apoptosis of HepG2 cells by activation of PPARγ, we pre-incubated HepG2 cells with GW9662, a selective antagonist of PPARγ, and observed the effect of ADFMChR on apoptosis and PPARγ and NF-κB protein expression of HepG2 cells. Our results showed that preincubation with GW9662 could effectively antagonize ADFMChR-induced apoptosis of HepG2 cells and down-regulation of NF-κB protein expression, suggesting that apoptosis of HepG2 cells induced by ADFMChR is dependent on activation of PPARγ.

Apoptosis is a complex process involving several genes, such as Bcl-2, Bax, and great attention has been given to the Bcl-2 family. The Bcl-2 family can positively and negatively regulate apoptosis[29]. Bcl-2 and Bax are two members of the Bcl-2 family, and play different roles in programmed cell death[30]. When Bax is over-expressed in cells, apoptosis in response to death signals is accelerated, leading to its designation as a death agonist[31]. When Bcl-2 is over-expressed it heterodimerizes with Bax and death is repressed[31]. Therefore, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis[30]. The results in this study confirmed that Bcl-2 expression in non-treated HepG2 cells was higher than in those treated with 3.0, 10.0, 30.0 μmol/L ADFMChR for 24 h; in contrast, Bax expression was lower. Thus, the ratio of Bcl-2 to Bax in HepG2 cells treated with ADFMChR was lower than that of non-treated HepG2 cells, which indicated that ADFMChR-induced HepG2 cells apoptosis was associated with down-regulation of Bcl-2 expression, up-regulation of Bax expression and reduction of the ratio of Bcl-2 to Bax.

In summary, ADFMChR possesses stronger anti-hepatic cancer effect *in vitro* than parent compound ChR, and was similar to 5-FU, and it exerts its apoptotic effect by activation of PPARγ, down-regulation of NF-κB and Bcl-2 protein expression, up-regulation of Bax protein expression, and reduction of the ratio of Bcl-2 to Bax. ADFMChR might be a promising candidate for the development of antitumor drugs.

COMMENTS

Background

Human liver carcinoma is the fifth most common cancer in the world. Unfortunately, the disease is often diagnosed at a late stage. For these patients, medical treatments, including chemotherapy, chemoembolization, ablation, and proton beam therapy, are not adequate. Most patients show disease recurrence that rapidly progresses to the advanced stages with multiple intrahepatic metastases and their 5-year relative survival rate is only 7%. Clearly, there is an urgent need for new therapies for this disease.

Research frontiers

Enhancing the antitumor effect of existing anticancer drugs, but not to increase its toxicity, is the aim of current anticancer research. Natural compounds have been extensively studied and have shown anti-carcinogenic activities by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis. Flavonoids are a group of polyphenolic substances widely distributed in the plant kingdom and present in human diets. Previous reports have shown that flavonoids (such as chrysin, apigenin) could inhibit the proliferation and induce apoptosis in tumor cells. In this study, the authors demonstrate that 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) could induce apoptosis of human liver carcinoma HepG2 cells *in vitro* by activation of PPARγ.

Innovations and breakthroughs

Recent research has shown that chrysin and its derivatives possess a strong anticancer effect. This is the first study to report that ADFMChR, the derivative of chrysin, has a greater suppressive effect on proliferation of HepG2 cells than that of chrysin, and induces apoptosis of HepG2 cells. These data support the idea that ADFMChR has great potential to be developed into novel cancer preventative agents.

Applications

This finding may provide a molecular basis for the clinically observed cancer-preventive effects of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) and new clues for research about pharmaceutical prevention and cure of human liver carcinoma.

Terminology

ADFMChR, a Chrysin derivative, which was taken as the principle compound to design and synthesize, was prepared by alkylation, methylation, and gen-difluoromethylation of chrysin, and was found to have stronger anticancer activities than parent compound chrysin.

Peer review

The authors demonstrate that the effects of ADFMChR on induction of apoptosis in HepG2 cells may be associated with activation of PPARγ, sequentially accompanied by inhibition of protein expression of NF-κB and Bcl-2 and reduced ratio of Bcl-2 to Bax. The results provide a new idea for cure of human liver carcinoma.

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BRIEF ARTICLES

No association between cyclooxygenase-2 and uridine diphosphate glucuronosyltransferase 1A6 genetic polymorphisms and colon cancer risk

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Abstract

AIM: To investigate the association of variations in the cyclooxygenase-2 (COX2) and uridine diphosphate glucuronosyltransferase 1A6 (UGT1A6) genes and non-steroidal anti-inflammatory drugs (NSAIDs) use with risk of colon cancer.

METHODS: NSAIDs, which are known to reduce the risk of colon cancer, act directly on COX2 and reduce its activity. Epidemiological studies have associated variations in the *COX2* gene with colon cancer risk, but others were unable to replicate this finding. Similarly, enzymes in the *UGT1A6* gene have been demonstrated to modify the therapeutic effect of NSAIDs on colon adenomas. Polymorphisms in the *UGT1A6* gene have been statistically shown to interact with NSAID intake to influence risk of developing colon adenomas, but not colon cancer. Here we examined the association of tagging single nucleotide polymorphisms (SNPs) in the *COX2* and *UGT1A6* genes, and their interaction with NSAID consumption, on risk of colon cancer in a population of 422 colon cancer cases and 481 population controls.

RESULTS: No SNP in either gene was individually statistically significantly associated with colon cancer, nor did they statistically significantly change the protective effect of NSAID consumption in our sample. Like others, we were unable to replicate the association of variants in the *COX2* gene with colon cancer risk ($P > 0.05$), and we did not observe that these variants modify the protective effect of NSAIDs ($P > 0.05$). We were able to confirm the lack of association of variants in *UGT1A6* with colon cancer risk, although further studies will have to be conducted to confirm the association of these variants with colon adenomas.

CONCLUSION: Our study does not support a role of COX2 and UGT1A6 genetic variations in the development of colon cancer.

Key words: Uridine diphosphate glucuronosyltransferase 1A6; Cyclooxygenase-2; Non-steroidal anti-inflammatory drugs; Colon cancer; Genetic association studies; Single nucleotide polymorphisms

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INTRODUCTION

Almost 150 000 new colorectal cancer cases are estimated to be diagnosed in 2008, resulting in almost 50 000 deaths (National Cancer Institute-www.cancer.gov). Colon adenomas (polyps) are a well established precursor of colon cancer. The genetic and environmental factors that cause the development of colon adenomas or their subsequent progression into cancer are not entirely known. Genetics are known to be a large risk factor for colon cancer, and indeed having a family history of colon cancer increases your risk of developing it yourself substantially. However, the known genetic susceptibility loci for colon cancer make up only a small fraction of this risk.

Cyclooxygenase-2 (COX-2) is a pro-inflammatory enzyme that converts arachidonic acid to prostaglandins. COX2 has been shown to be upregulated in a high proportion (about 86%) of human colorectal cancer[1]. Numerous additional findings have indicated a likely role for the cycloxygenases and inflammation in the development of colon cancer[2].

Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and ibuprofen, act directly on COX2 as well as other targets to reduce activity. A substantial body of epidemiologic and randomized clinical trial evidence suggests that regular NSAID use and selective COX-2 inhibitors reduce the risks of colorectal cancer or the recurrence of adenomatous polyps[3], which is, at least in part, attributed to their known anti-inflammatory effects. The *COX-2* gene is thus a good candidate gene for colorectal carcinogenesis and its genetic variants may affect the susceptibility to the development of this colorectal cancer by altering the effects of this enzyme on the inflammatory response. To date, a few studies have evaluated a limited number of polymorphisms in the *COX2* gene in relation to risk of colorectal cancer and adenomatous polyps[4-7]. The findings have yielded inconsistent results, with some[4-6], but not all[7], reporting an association with the risk of colorectal cancer or polyps. Additionally, a sib-pair study of colon neoplasia with relatively small sample size found no linkage to the *COX2* locus[8].

Furthermore, genetic variation in the uridine diphosphate glucuronosyltransferase 1A6 (*UGT1A6*) gene have been associated with differences in aspirin metabolism, and those with the less frequent variants have a 30%-50% lower enzyme activity[9]. Additionally, a few studies have examined the association of genetic polymorphisms in *UGT1A6*, a rate-limiting enzyme directly involved in aspirin metabolism, and their interactions with aspirin or NSAIDs in relation to colorectal cancer and polyps[10-13]. Although two studies of colon cancer observed no association with genetic variants in *UGT1A6*[12,13], two studies of colon adenomas identified variants in *UGT1A6* that modified the protective effect of aspirin[10,11]. Others have identified variants that influence the risk of adenoma recurrence[14]. To further investigate the role of *COX2* and *UGT1A6* in relation to the risk of colon cancer, we tested nine tag single nucleotide polymorphisms (SNPs) in *COX2* and two functional SNPs in *UGT1A6*[13] in a population-based case-control study of colon cancer.

MATERIALS AND METHODS

Study population

The details of the present study have been described elsewhere[15]. Briefly, colon cases (n = 422) were identified through the population-based Surveillance, Epidemiology and End Results (SEER) Kentucky Cancer Registry, and 481 population controls living in Kentucky were recruited *via* random digit dialing. Participants donated a blood sample for genetic analyses and completed a detailed self-administered lifestyle risk factor questionnaire that included information on NSAID use. Participation rates were 72.2% for cases and 62.5% for controls. All participants provided written informed consent. The study was approved by the Institutional Review Boards of the University of Kentucky, Lexington, and Case Western Reserve University/University Hospitals Case Medical Center.

Genotyping

We assessed the common genetic variation (SNPs with minor allele frequencies > 5%) of *COX-2* that spanned about 2 kb upstream of the transcription start site and about 1 kb downstream of the 3' untranslated region. Seven tag SNPs for COX-2 were selected to predict unmeasured SNPs ($r^2 > 0.8$) using publicly available genotype data for European populations from the International HapMap project (www.hapmap.org) and the Perlegen and Seattle SNP projects (http://gvs.gs.washington.edu/GVS). One common SNP, -899 G/C (rs20417), that was previously associated with colorectal cancer[9], and rs689470, which was previously associated with prostate cancer[16], were also included. Two functional non-synonymous SNPs [rs1105879 (R184S) and rs2070959 (T181A)] in UGT1A6[13] were selected. Genotyping was performed using the Taqman allelic discrimination assay with genotyping error < 0.1%, as described previously[15].

Statistical analyses

We evaluated the association between *COX-2* and *UGT1A6* genotypes and colon cancer risk using multivariate unconditional logistic regression models. Each SNP was evaluated assuming dominant, additive and recessive modes of inheritance. For all SNPs, the allele with the lower frequency was coded as the risk allele. For the dominant model, individuals with at least one copy of the risk allele were coded as 1, others were

coded as 0. For the recessive model, only those with two copies of the risk allele were coded as 1. In the additive model, individuals were coded with the number of risk alleles they possessed (0, 1 or 2). In our base models, we adjusted for age, gender, and race. For these analyses, age was defined as age at diagnosis for cases and age at recruitment for controls. We further controlled for family history of colorectal cancer, body mass index (BMI), regular NSAID use, alcohol consumption, smoking, and intensity of recreational physical activity in the 20 s, for those with available data. Regular NSAID use was defined as having reported intake of at aspirin or ibuprofen at least twice a week for a period of one month or longer.

To evaluate potential effect modification of NSAID use, we tested for multiplicative interaction by including the main effects and a cross-product term of SNP × NSAID use in the logistic regression models. All *P*-values were two-sided, and all analyses were undertaken with SAS software (version 9.1; SAS Institute, Inc., Cary, North Carolina).

RESULTS

The characteristics and genotypic distributions of this predominantly Caucasian study population are summarized in Table 1. All SNPs were found to be in Hardy-Weinberg equilibrium both in controls alone and in the entire sample. We found no statistically significant association between any of the nine *COX2* SNPs and two functional *UGT1A6* SNPs and colon cancer risk, regardless of the mode of inheritance (Table 2).

We further explored potential effect modification of the association by regular NSAID use, and found no evidence for interaction of any SNP (Table 2) in either gene ($P > 0.05$).

Since others[10] have reported that UGT1A6 variants modify the therapeutic effects of aspirin in a population of women only, we stratified our analyses by gender. We found very little differences in results with nothing significant in females or males only as well (results not shown).

DISCUSSION

In our analysis, we examined potential effect modification by regular NSAID use rather than aspirin alone, as other groups have done, due to the smaller number of aspirin alone users. To account for this, we repeated our analysis using aspirin use only (115 cases and 157 controls), and the results did not change materially (not shown), with no significant findings. However, the lack of association with aspirin alone may be due to the small sample size and thus lack of statistical power.

Our study had over 90% power to detect an OR ⩾ 1.75 at a two-sided $\alpha = 0.05$ for the polymorphisms studied here, and over 80% power to detect an OR ⩾ 1.60, assuming a dominant model of inheritance and allele frequency of 0.1. While we did comprehensively capture the common genetic variation across the *COX2* gene, we only evaluated two putative functional SNPs in *UGT1A6*, and were limited in our ability to make direct conclusions about the effect of other genetic variants in *UGT1A6*. It is possible that these variants have smaller effects on colorectal cancer susceptibility or the therapeutic effects of NSAID use that we were unable to detect with this study.

Table 1 Population characteristics *n* (%)

	Case (*n* = 422)	Control (*n* = 481)	*P*[1]
Age (mean ± SD) (yr)[2]	62.9 ± 10.6	57.9 ± 11.1	< 0.0001
Gender			0.0002
Female	203 (50.5)	304 (64.1)	
Male	199 (49.5)	178 (35.9)	
Race			
Caucasian	378 (93.6)	449 (93.2)	0.35
African-American	21 (4.4)	21 (5.2)	
Other	12 (2.5)	5 (1.2)	
BMI (mean ± SD) (kg/m^2)	29.2 ± 6.2	28.1 ± 6.1	< 0.0001
Family history[3]			0.0011
Yes	94 (24.0)	71 (15.2)	
No	297 (76.0)	395 (84.8)	
NSAID use			0.14
Regular	235 (64.2)	306 (69.1)	
Irregular/none	131 (35.8)	137 (30.9)	
Physical activity			0.008
None/low	111 (29.1)	113 (24.7)	
Moderate	106 (27.8)	98 (21.4)	
High	165 (43.2)	246 (53.8)	
Regular alcohol use			0.04
Ever	134 (34.8)	191 (41.7)	
Never	251 (65.2)	267 (58.3)	
Smoking			0.88
Ever	207 (53.6)	248 (54.2)	
Never	179 (46.4)	210 (45.9)	

[1]*P*-value of significance difference between cases and controls in χ^2 test (discrete variables and genotypes) or *t*-test (continuous); [2]Age at diagnosis for cases, and age at questionnaire completion for controls; [3]Family history of first-degree relatives with colorectal cancer.

It is important to note that NSAID use was based on self-report. Individuals may not accurately recall duration or frequency of NSAID intake. Nevertheless, our finding of a protective effect of NSAID use (OR = 0.69, 95% CI = 0.50-0.96, $P = 0.02$) is in agreement with its well-documented association with colon cancer[17], lending credibility to our questionnaire data.

In conclusion, this moderately large population-based case-control study did not detect a direct association between variants in the *UGT1A6* and *COX2* genes and risk of colon cancer nor an effect modification by NSAIDs. The results of our study are in line with the two studies of colon cancer showing null results[12,13]. Taken together with the studies showing an association with polyps[10,11], our results suggest genetic variation of *UGT1A6* may affect the early stages of colon tumorigenesis, but has little influence on the progression from adenomatous polyps to colon cancer, although we are unable to test that hypothesis directly.

Table 2 Odds ratios for individual SNPs and SNP by NSAID use interactions

SNP	Case/control	Crude		Adjusted		Stratified by NSAID use		
		OR (95% CI)	P^1	OR (95% CI)	P^2	Regular	None	P^3
COX2								
rs2066826			0.45		0.34			0.41
GG	314/370	Ref		Ref		Ref	Ref	
AG	98/99	1.17 (0.85, 1.60)		1.23 (0.88, 1.72)		1.05 (0.68, 2.64)	1.52 (0.82, 2.83)	
AA	8/12	0.79 (0.32, 1.95)		1.06 (0.41, 2.73)		0.52 (0.10, 2.64)	1.96 (0.30, 12.62)	
rs2206593			0.049		0.25			0.47
GG	382/417	Ref		Ref		Ref	Ref	
AG	39/63	0.68 (0.44, 1.03)		0.69 (0.44, 1.07)		0.64 (0.36, 1.13)	0.89 (0.42, 1.90)	
AA	0/2	----		----		----	----	
rs5277			0.97		0.58			0.76
CC	310/353	Ref		Ref		Ref	Ref	
CG	104/119	1.00 (0.73, 1.35)		0.90 (0.65, 1.25)		0.90 (0.59, 1.36)	0.86 (0.48, 1.54)	
GG	7/8	1.00 (0.36, 2.78)		0.80 (0.27, 2.36)		0.52 (0.09, 2.95)	1.85 (0.32, 10.56)	
rs689470			0.87		0.79			> 0.99
GG	380/440	Ref		Ref		Ref	Ref	
AG	35/36	1.13 (0.69, 1.83)		1.15 (0.66, 2.02)		1.13 (0.54, 2.35)	1.59 (0.60, 4.22)	
AA	4/4	1.16 (0.29, 4.66)		1.60 (0.26, 9.70)		----	2.38 (0.13, 43.43)	
rs4648310			0.95		0.67			0.50
AA	405/462	Ref		Ref		Ref	Ref	
AG	17/19	1.08 (0.55, 2.12)		1.07 (0.53, 2.19)		1.25 (0.48, 3.23)	0.69 (0.21, 2.27)	
GG	0/1	----		----		----	----	
rs5275			0.34		0.51			0.89
AA	176/216	Ref		Ref		Ref	Ref	
AG	189/199	1.17 (0.88, 1.55)		1.17 (0.87, 1.58)		1.14 (0.78, 1.67)	1.04 (0.60, 1.79)	
GG	56/65	1.06 (0.70, 1.59)		1.22 (0.79, 1.88)		0.98 (0.57, 1.71)	1.79 (0.78, 4.12)	
rs689466			0.46		0.69			0.99
AA	275/297	Ref		Ref		Ref	Ref	
AG	138/168	0.89 (0.67, 1.17)		0.86 (0.64, 1.15)		0.91 (0.62, 1.33)	0.83 (0.48, 1.42)	
GG	9/15	0.65 (0.28, 1.51)		0.65 (0.26, 1.60)		0.75 (0.26, 2.17)	0.69 (0.11, 4.45)	
rs20417			0.41		0.20			0.44
GG	291/343	Ref		Ref		Ref	Ref	
CG	119/121	1.16 (0.86, 1.56)		1.25 (0.91, 1.71)		1.17 (0.78, 1.74)	1.39 (0.78, 2.47)	
CC	11/15	0.86 (0.39, 1.91)		0.97 (0.43, 2.20)		0.46 (0.14, 1.54)	3.12 (0.57, 17.15)	
rs2745557			0.61		0.41			0.18
GG	287/321	Ref		Ref		Ref	Ref	
AG	120/141	0.95 (0.71, 1.27)		0.93 (0.69, 1.27)		1.04 (0.70, 1.54)	0.77 (0.43, 1.38)	
AA	13/19	0.76 (0.37, 1.58)		1.01 (0.48, 2.14)		1.49 (0.60, 3.67)	0.34 (0.07, 1.68)	
UGT1A6								
rs1105879			0.46		0.28			0.93
AA	191/206	Ref		Ref		Ref	Ref	
AC	167/209	0.86 (0.65, 1.14)		0.84 (0.63, 1.14)		0.84 (0.57, 1.23)	0.86 (0.50, 1.48)	
CC	64/66	1.05 (0.70, 1.55)		1.07 (0.71, 1.64)		1.20 (0.70, 2.06)	1.07 (0.50, 2.32)	
rs2070959			0.21		0.21			0.93
AA	208/207	Ref		Ref		Ref	Ref	
AG	154/206	0.78 (0.59, 1.03)		0.78 (0.58, 1.04)		0.76 (0.52, 1.12)	0.82 (0.48, 1.42)	
GG	59/57	1.08 (0.72, 1.63)		1.11 (0.72, 1.72)		1.26 (0.72, 2.23)	1.04 (0.47, 2.29)	

[1]*P*-value of SNP in best fitting genetic model in the unadjusted logistic regression; [2]*P*-value of SNP in best fitting genetic model in the full multivariate logistic regression; [3]*P*-value of interaction of SNP in best fitting genetic model with regular NSAID use in the logistic regression.

COMMENTS

Background

Colon cancer accounts for almost 150 000 cancer cases and 50 000 deaths in the United States alone. Non-steroidal anti-inflammatory drug (NSAID) use has been well known to reduce the risk of colon cancer.

Research frontiers

It is unclear how individual variation influences the protective effect of NSAIDs. Cyclooxygenase-2 (*COX2*) and uridine diphosphate glucuronosyltransferase 1A6 (*UGT1A6*) are two genes that have been proposed to modify the effect of NSAIDs on colon cancer risk. *COX2* is a direct target of NSAIDs and UGT1A6 variations have been shown to alter the metabolism of aspirin, a common NSAID.

Innovations and breakthroughs

This study has provided further insight into the role of the *COX2* and *UGT1A6* genes in colon cancer risk.

Applications

Since genetic variation often accounts for differences in an individual's response to preventive or therapeutic drugs, it is important to understand the relationship between genes, the drugs and the intended outcome. NSAIDs have been suggested as a chemopreventive agent for individuals at high risk of colon cancer. It is thus important to identify those individuals who would most benefit from the use of NSAIDs. This study found that, while other genes may predict enhanced benefit of NSAID use for colon cancer prevention, *COX2* and *UGT1A6* do not.

Terminology

COX2 and *UGT1A6* are two genes involved in NSAID metabolism. Since NSAIDs, including aspirin and ibuprofen, are known to be protective for colon cancer, it has been hypothesized that these genes would influence and individual's response to NSAID use with respect to colon cancer risk.

Peer review

The authors examined the association of *COX2* and *UGT1A6* polymorphisms

and risk of colon cancer. They also evaluated the effect of variations in these genes on the protective effect of NSAIDs. They did not find an association with any variant examined and risk of colon cancer nor did they find they these variants altered NSAID effects. This study gives further evidence that these genes are not directly involved in colon cancer carcinogenesis.

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Contrast-enhanced sonography *versus* biopsy for the differential diagnosis of thrombosis in hepatocellular carcinoma patients

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Abstract

AIM: To clarify which method has accuracy: 2nd generation contrast-enhanced ultrasound or biopsy of portal vein thrombus in the differential diagnosis of portal vein thrombosis.

METHODS: One hundred and eighty-six patients with hepatocellular carcinoma and portal vein thrombosis underwent in blinded fashion a 2nd generation contrast-enhanced ultrasound and biopsy of portal vein thrombus; both results were examined on the basis of the follow-up of patients compared to reference-standard.

RESULTS: One hundred and eight patients completed the study. Benign thrombosis on 2nd generation contrast-enhanced ultrasound was characterised by progressive hypoenhancing of the thrombus; in malignant portal vein thrombosis there was a precocious homogeneous enhancement of the thrombus. On follow-up there were 50 of 108 patients with benign thrombosis: all were correctly diagnosed by both methods. There were 58 of 108 patients with malignant thrombosis: amongst these, 52 were correctly diagnosed by both methods, the remainder did not present malignant cells on portal vein thrombus biopsy and showed on 2nd generation contrast-enhanced ultrasound an inhomogeneous enhancement pattern. A new biopsy during the follow-up, guided to the area of thrombus that showed up on 2nd generation contrast-enhanced ultrasound, demonstrated an enhancing pattern indicating malignant cells.

CONCLUSION: In patients with hepatocellular carcinoma complicated by portal vein thrombosis, 2nd generation contrast-enhanced ultrasound of portal vein thrombus is very useful in assessing the benign or malignant nature of the thrombus. Puncture biopsy of thrombus is usually accurate but presents some sampling errors, so, when pathological results are required, 2nd generation contrast-enhanced ultrasound could guide the sampling needle to the correct area of the thrombus.

Key words: Hepatocellular carcinoma; 2nd generation contrast enhanced ultrasound; Contrast enhanced sonography; Malignant thrombosis; Portal vein biopsy

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INTRODUCTION

About 20% of patients at first access visit to a specialized centre for the care of hepatocellular carcinoma (HCC)[1] are in need of differential diagnosis between be-

nign portal vein thrombosis (PVT) or malignant thrombosis. The nature of the thrombus can have a significant impact on treatment. In particular, because the prevalence of tumor recurrence is nearly 100%, patients who have HCC and proven neoplastic vascular thrombus are not candidates for any treatment[2-5]. Malignant PVT can occur in patients with cirrhosis, with or without the presence of parenchymal HCC, because there is the possibility of intravascular first growth of this neoplasm[6]. Thrombi have been studied in an effort to determine imaging characteristics that could be used to distinguish benign from malignant thrombi[7-11]. Unfortunately, the imaging characteristics tend to overlap, in particular on computer tomography (CT) or magnetic resonance imagine (MRI) exams only the feature of thrombus-tumor continuity is widely accepted as a reliable indicator of thrombus malignancy[12,13]. In patients where percutaneous ablation of HCC is the therapy of choice, the technique of reference throughout the world for differentiating benign from malignant PVT is percutaneous fine needle biopsy (FNB) of the thrombus[14]. Given the obvious clinical utility of a reliable non-invasive technique for diagnosis of malignant PVT, the limitation of previous imaging studies and an opportunity at our institutions to perform a reasonably large prospective study with cytopathologic correlation in all patients, we undertook an investigation to compare Contrast-Enhanced Sonography (CEUS) and portal vein FNB of thrombus in differentiating benign from malignant thrombosis.

MATERIALS AND METHODS

The study protocol which was fully concordant with ethical principles of the Declaration Helsinki was approved by the institutional ethic committee. A written informed consent was obtained from each patient.

Patients

From January 2001 to February 2006, we enrolled consecutively 256 cirrhotic patients with HCC and PVT (Table 1). The major part of these patients were not eligible for surgical resection/liver transplantation, the others refused intervention. We restricted analysis only to patients without direct contiguity between the thrombus and HCC and considering also the patients drop out on follow-up we completed the study in 108 patients. Clinical and ultrasonography (US) details of these patients are displayed in Table 2.

Study design

Diagnosis of HCC was made according to the guidelines drawn up the Barcelona 2000 EASL Conference[15]. These guidelines suggest that in a liver cirrhosis setting HCC may be diagnosed by coincidental findings in at least two imaging modalities (spiral CT and Doppler US or MRI) that should reveal arterial hypervascularity or in the case of combined criteria (spiral CT with alfa-fetoprotein levels > 400 ng/mL). US guided biopsy should be performed in those cases in which the above-mentioned criteria are not satisfied[15]. Pathological diagnosis of HCC was made according to the International Working Party criteria[16]. The thrombi were detected on routine sonographic and CT examination. Spiral CT was performed in a range of one month before or after color Doppler US. In patients after diagnosis of HCC, in order to characterize PVT, we performed both CEUS and portal FNB; according to the results of portal FNB patients were evaluated for potential percutaneous ablation of HCC. Study design is displayed in Figure 1. Patients underwent first CEUS then portal FNB on the same occasion carried out by two separate operators; the operator that performed PVT FNB was blinded to the results of CEUS. Patients without malignant cells on FNB underwent percutaneous treatment; the others underwent supportive care. Results of baseline CEUS were evaluated in blind fashion on the basis of the evolution

Table 1 Enrollment design

Contents	
Inclusion criteria	Presence of one to three focal HCC Presence of intra-vascular[1] portal vein thrombosis Child-Pugh class A or B
Patients initially enrolled	Men: 190 Women: 66
Excluded for US evidence direct HCC portal vein invasion	70
Drop out on follow-up	78 (42%)
Died	30
Liver could not be adequately visualized	9
Patients studied	Mean age: 66 ± 6 Men: 82 Women: 26

[1]Not US features of infiltration of perivascular parenchyma: intact vessel wall.

Table 2 Principal clinical/ultrasound features of patients

Clinical data	Results
Child A/B	44/64
Etiology	
HCV related	58
HBV related	23
Alcohol related	12
Mixed etiology	15
Number of HCC nodules	
Single nodule	10
Median size	44 mm (range 40-75 mm)
Two nodules	22
Median size	41 mm (range 33-68 mm)
Three nodules	76
Median size	39 mm (range 32-67 mm)
Topography of portal vein thrombosis	
In right or left but not in the main portal vein	80 (74%)
In main, right and left portal vein	14 (13%)
In right or left and main portal vein	12 (11%)
In main portal vein	2 (1.8%)
Complete or incomplete vessel occlusion of on power-color-Doppler	
A complete occlusion of the portal vessel	99 (91%)
Incomplete thrombosis lumen	9 (9%)

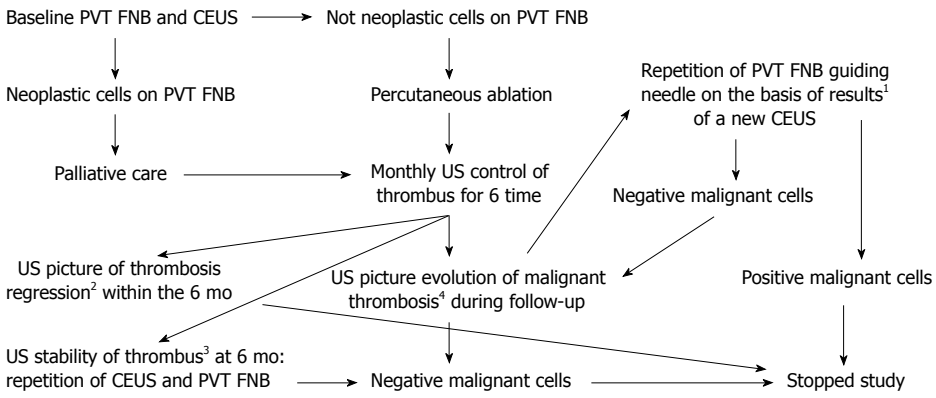

Figure 1 Study design. PVT FNB: Portal vein thrombus fine needle biopsy; CEUS: 2nd generation Contrast-Enhanced US (CEUS) of thrombus; [1]Guiding needle on portion of thrombus showing on CEUS precocious iso or hyperenhancement pattern; [2]No increase in size and distribution with preservation of vessel wall or recanalization/shrinkage, or disappearance of a PVT within the 6 mo of follow-up were accepted as proof of a benign portal vein thrombus; [3]No change in feature of thrombus and in the diameter of the segment of involved vein at 6 mo of follow-up; [4]Increase in size with infiltration of perivascular parenchyma and interruption of vessel wall was US features indicative of malignant thrombosis.

of thrombus on follow-up and were not decisive for the therapeutic management of patients. All patients after CEUS and PVT FNB were followed up for 6 mo; they underwent monthly US examination by an operator that was blinded to CEUS and PVT FNB initial results. We considered as the reference standard of benign or malignant thrombosis the US evolution of thrombus in combination with concordant cytology on new PVT FNB: i.e. no increase in size and distribution with preservation of vessel wall or recanalization/shrinkage, or disappearance of a PVT within the sixth months of follow-up were accepted as proof of a benign portal vein thrombus. However, in cases of stability of thrombi with no change in diameter of the segment of involved vein at 6 mo of follow-up, patients were resubmitted to CEUS and PVT FNB; in absence of malignant cells at this cytological examination, thrombus was definitively considered benign. Our reference standard of malignant thrombosis was increase in size on US, with or without infiltration of perivascular parenchyma and interruption of vessel wall at any time point during the follow-up. In the presence of such evolution of the US picture, CEUS and PVT FNB were repeated with guidance of the needle biopsy to thrombus areas with enhancing pattern allowing for the search for malignant cells; in presence of these, patients stopped the follow-up and thrombus was definitively considered malignant. Patients that died on follow-up without definitive diagnosis were considered drop outs. Specimens were obtained with a 22-Gauge Chiba needle in all patients; needles are manufactured with a removable occlusive stylet. The same biopsy technique described by others[14] was used in all patients. A positive result was considered if the biopsy specimen contained hepatocytes that had malignant features.

Baseline and contrast-enhanced harmonic ultrasound

An Aloka-Prosounds-5500-model equipped with a multifrequencies 2-6 MHz sector probe, was used. Contrast-enhanced imaging was performed according to the protocol used for the Bracco-SonoVue preclinical trial[17]. Examination was performed with low acoustic power (mechanical index under 0.01). SonoVue (BR1; Bracco, Milan, Italy)[18,19] consisted of sulfur-hexafluoride (SF_6) vapor-filled and phospholipid-stabilized microbubbles with a diameter uniformly smaller than 8 μm; these microbubbles circulate in the intravascular space crossing pulmonary and systemic capillary circulation[20,21]. 2.5 mL of contrast-agent were administered for each patient. Thanks to its ability to avoid destruction of bubbles, the low mechanical index technique allows identification of the entire vascular phase of contrast agent perfusion, consisting of the arterial phase (15-30 s after injection of agent), the portal phase (30-60 s after injection of agent) and the late parenchymal phase[22-24]. Positive arterial enhancement of the thrombus was defined as a greater hyperechogenicity of the vascular bed-occupying lesion in comparison to the surrounding liver parenchyma detected during the arterial phase. Two independent highly experienced readers firstly performed off-site assessments of the videotapes in a computer-generated randomised order. The readers were blinded to all clinical and pathological information as to the nature of the analysed thrombi.

Statistical analysis

Sensitivity, specificity, positive and negative predictive values of CEUS and PVT FNB were obtained for diagnosis of the nature of the thrombus; we considered as reference standard the US picture evolution of thrombus on follow-up, with a new PVT FNB as above decrypted accordingly to obtain definitive cytological confirmation.

RESULTS

On follow-up we identified 58 of 108 patients (53.7%) with malignant thrombosis and 50 (46.3%) with benign thrombosis. Figure 2 displayed results of combined tests: in 50 of 56 patients without malignant cells on first PVT FNB, benign PVT was characterized on CEUS by a diffuse homogeneous hypoenhancing pattern and this appearance was persistent compared with the adjacent liver, also during late phase (Figure 3A-C). In the follow up of

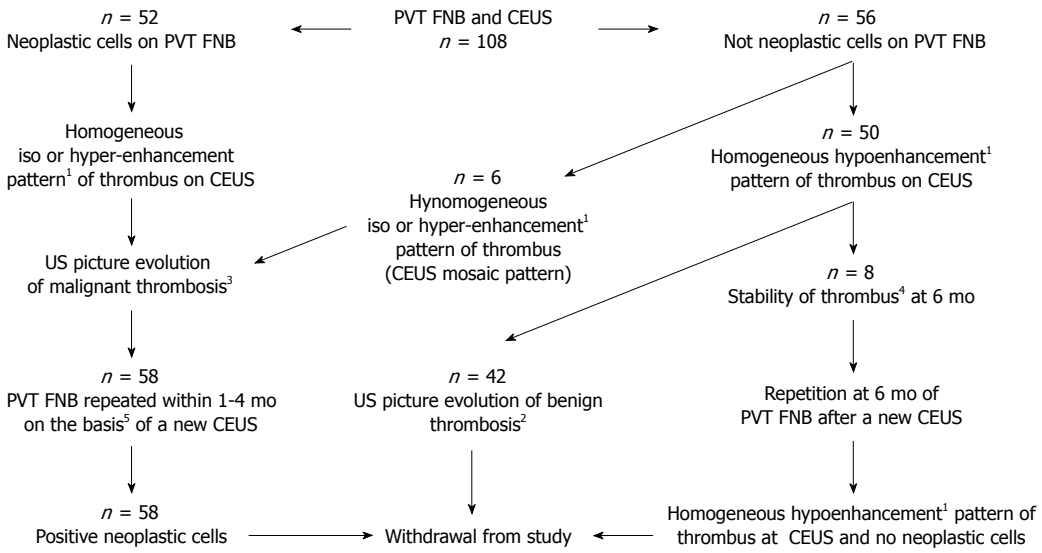

Figure 2 Summary of combined test results. [1]Iso, hyper, or hypoenhancement pattern of thrombus compared to the surrounding parenchyma; [2]Reference standard of benign thrombosis is a US evidence of evolving thrombus: no increase in size or distribution with vessel wall preservation or recanalization/shrinkage, or disappearance of a PVT within the 6 mo of follow-up were accepted as evidence of a benign portal vein thrombus; [3]Us image of evolution, indicating malignant thrombosis: increase in size with infiltration of perivascular parenchyma and interruption of vessel wall was US features of malignant thrombosis; [4]No change in thrombus image and in the diameter of the segment of vein involved at 6 mo of follow-up; [5]PVT FNB were repeated guiding the needle to the thrombus territories with enhancing pattern.

these patients we observed 16 spontaneous disappearances of thrombi after treatment of HCC, 26 recanalization with shrinkage of thrombi and 8 cases of stability of thrombi with no change in diameter of the segment of involved vein. These benign PVT patients were resubmitted to CEUS and PVT FNB at 6 mo with the same combined results as at the start (Figure 2). In 6 patients of 56 without malignant cells on FNB (false negative on PVT FNB), CEUS showed no homogeneous arterial enhancement of some small portions of thrombus. On follow-up the thrombus of these patients showed intravascular spread with growth in maximal diameter of the involved segments of the portal branch from a mean of 8 mm to a mean of 14 mm, with interruption of the vessel wall in 3 patients; we repeated CEUS and guided a new portal FNB to areas of thrombus that showed an enhancing pattern, obtaining positive results for malignant hepatocytes. The islands of neoplastic tissue were located at baseline CEUS as corresponding to the anterior wall of right branch in 1 case, corresponding to and mainly in the centre of the vessel in 3 cases, and corresponding to the posterior wall of left portal branch in 2 cases; they measured between 9 mm and 15 mm in length. In all 6 cases there was a complete thrombosis, involving the portal trunk and both branches, which measured in length between 18 mm and 27 mm. We retrospectively called the CEUS appearance of these cases "mosaic picture" of neoplastic thrombus (Figure 4A and B).

There were 52 patients (Figure 2) with the presence of malignant cells on the baseline portal FNB: these showed on follow-up growth in diameter and intravascular spread of PVT within 1-4 mo. The repetition of PVB FNB in all these patients, with an US picture evolution of malignant thrombosis on the basis of a new CEUS, always confirmed the diagnosis (not false-negative). Typical malignant PVT had an unequivocal appearance at CEUS: during the arterial phase intense and diffuse homogeneous contrast enhancement (Figure 5A and B) was seen, followed or not by a washout of contrast material from the thrombus; the appearance was iso or hyperechoic in arterial phase and hypo or isoechoic during the late phase (Table 3). Sensitivity, specificity, positive and negative predictive value of PVT FNB and CEUS were the same for both, respectively: 89.6%, 100%, 100%, 89.2%. These

Table 3 Contingent tables

Group	Results
Patients studied on follow-up	108
Malignant thrombosis	58 (53.7%)
Benign thrombosis	50 (46.3%)
Presence of neoplastic cells on PVT FNB[1]	
True positive	52 (48.1%)
False positive	0
Not neoplastic cells on baseline PVT FNB	
False negative	6[2] (5%)
True negative	50 (46.3%)
Iso-hyper-enhancement pattern[1] on CEUS and mosaic pattern	
True positive	58
Precocious isoenhancement pattern	21
Precocious hyperenhancement pattern	31
Mosaic pattern[3]	6
False positive	0
Hypoenhancement patternc on CEUS	
False negative	0
True negative	50 (46.3%)

Sensitivity, specificity, positive and negative predictive value of CEUS: All 100%. [1]Iso-hyper-hypoenhancement pattern of thrombus respect to surround parenchyma; [2]False negative patients on portal vein FNB were the same with mosaic pattern on CEUS; [3]Hynomogeneous iso-hyperenhancement of thrombus.

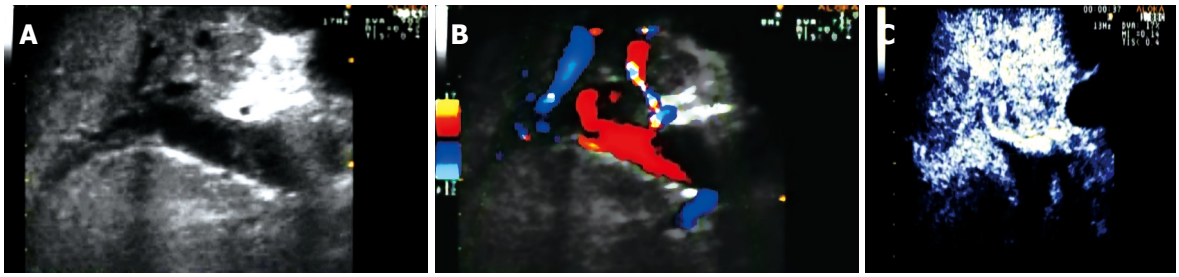

Figure 3 Benign thrombus. A: On sonography lumen of portal vein is partially filled with hypoechoic material representing occlusive thrombus; B: Color Doppler ultrasound reveals color signals only within a portion of portal lumen; C: Contrast-enhanced sonography scan during portal phase reveals uniformly non-enhancing area within portal vein, perfectly reproducing the benign thrombus.

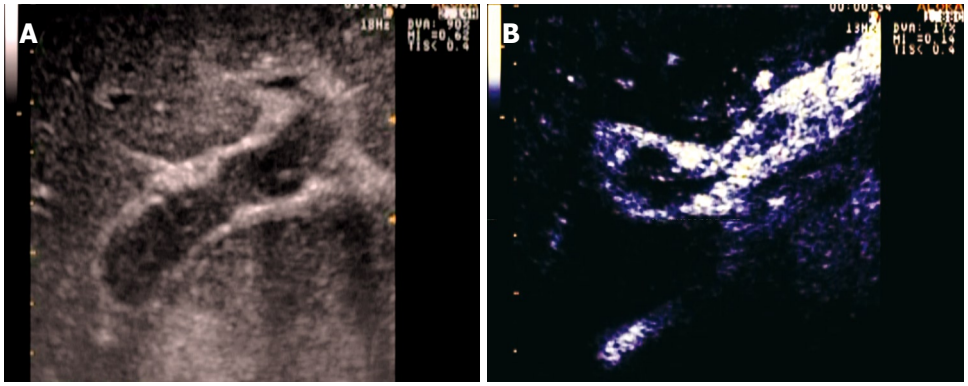

Figure 4 Malignant mosaic thrombus. A: Sonography scan reveals isoechoic area within portal lumen representing thrombus; B: Contrast enhanced sonography scan during late arterial phase reveals thrombus as predominantly enhancing area, indicative of arterial neovascularization (malignant thrombosis) with some non-enhancing areas of the thrombus (mosaic pattern).

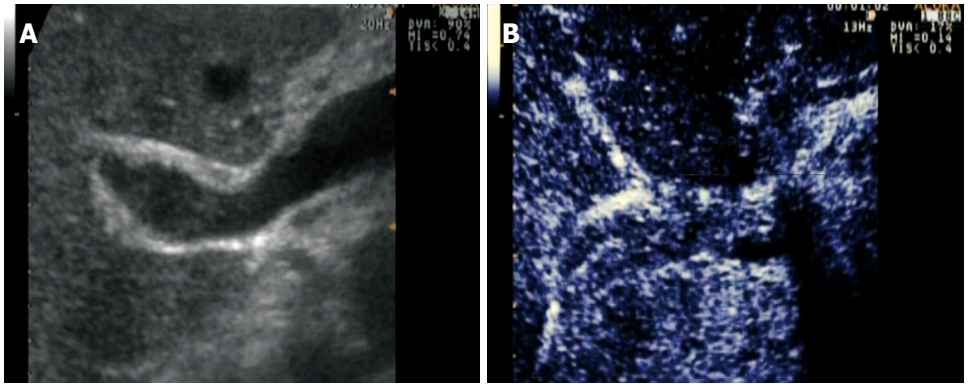

Figure 5 Malignant thrombus. A: sonography reveals echogenic area (thrombus) within vessel lumen; B: during arterial phase of contrast-enhanced sonography the diffusely enhanced area representing thrombus with internal neovascularity.

values coincided for both techniques because, as shown in Table 3, the false-negative patients on baseline CEUS and PVT FNB were the same. On the other hand, if we retrospectively admitted the mosaic picture of enhancement (the picture of the 6 false-negative patients on CEUS) as an alternative, but possible, picture of appearance of malignant PVT on CEUS, and considering that prospectively no false-positive or false-negative results were given, 100% of sensitivity and specificity were obtained for this technique.

DISCUSSION

In previous studies, we[25] and others[26] have described the usefulness and superiority of contrast-enhanced sonography with respect to sonography and color Doppler sonography in the detection and characterisation of thrombus. Here our study differs in two points: (1) we systematically compared in blinded fashion the validity of portal FNB with respect to contrast enhancement of portal thrombus; (2) we excluded from our study patients with evidence of continuity between thrombus and tumor tissue (most of patients in study of Rossi *et al*[26]) a feature considered diagnostic of malignant thrombosis both on sonography[26], and on helical TC/MRI imaging[12,13].

CT remains the primary imaging technique for staging HCC and identifying PVT[27]. MRI also appears to be a promising tool[28]. Although the capacity for CT to show main or lobar PVT is well established, controversy surrounds radiologists' ability to use CT to consistently differentiate between malignant and simple thrombi[3,7,27]. This reluctance to stage possible portal vein invasion by CT/MRI alone has perhaps been appropriate given the lack of a formal study in the literature that compares the imaging characteristics of proven benign and malignant thrombi. It was shown that tumor thrombus neovascularity may also be identified, with variable accuracy, by color Doppler

sonography[29-34]. Now the use of CEUS permits us to study in real time micro-vascular architecture of each thrombus, searching for global arterial enhancement typical of HCC neovascularity. The interpretation of results is based on general characteristics of enhancing/hypoenhancing of thrombus after administration of contrast ultrasound agent. The sensitivity of CEUS is better with respect to Doppler sampling of intrathrombus vessels; there are in fact technical limits of Doppler sampling due to the small diameter of vessels of the microvascular architecture of neoplastic tissue[35].

We deduced that homogeneous hypoenhancement of the thrombus on CEUS with respect to the surrounding parenchyma is diagnostic for benign thrombosis. Significantly, benign PVT does not show enhancement at any time after ultrasound contrast agent administration. The homogeneous enhancement of thrombus on CEUS must be considered diagnostic for malignant thrombosis. In particular, the appearance of malignant PVT can be precociously hyperechoic or isoechoic with respect to the surrounding parenchyma: this picture could be due to diffuse arterialization of surrounding liver parenchyma, a pathophysiological phenomenon secondary to the same thrombosis.

In our study, in order to obtain an accurate differential diagnosis as to the nature of a PVT, we utilized as gold-standard methods the prospective evaluation of the thrombus with, in most cases, a concordant cytology on PVT FNB repetition. We in fact were uncertain about the validity of using only baseline portal FNB to determine diagnosis because of the not optimal sensitivity of the method. The possibility of sampling error could result in both false-positive and false-negative diagnoses for malignant PVT. Because a benign thrombus does not contain hepatocytes, specimens that include cells from the periportal hepatic parenchyma or hepatocytes picked up during passage of the biopsy needle through the liver could lead to false-positive diagnoses of malignant tumor. We prevented false-positive diagnoses by using a biopsy needle with an occlusive stylet, keeping the stylet tightly seated until the needle tip was detected inside the portal vein and performing the biopsy under continuous sonographic visualization with the needle tip kept within the lumen of the portal vein at all times during passages. In our study no diagnosis of malignant tumor on FNB PVT was false-positive indicating that the invasive procedure is maximally specific. False-negative diagnoses for malignant cells could be produced if the portion of a malignant portal vein thrombus from which a specimen was obtained failed to contain malignant hepatocytes. We tried to prevent false-negative diagnoses by performing the biopsy on the portal vein thrombus by sampling the longest possible segment of a portal vein thrombus. We obtained anyway 6 false-negative results for malignant thrombi. In all 6 cases the appearance on CEUS was as an inhomogeneous enhancement of the thrombus; we called the CEUS appearance of these cases "mosaic-picture" of neoplastic thrombus. We were unhappy about the false-negative results of portal FNB derived from sampling the non-neoplastic portion of the thrombus; we repeated portal FNB within 1-4 mo guiding the biopsy on results of the CEUS (FNB of enhancing part of thrombus) and obtained malignant cells. We supposed that the echotexture of malignant thrombus on CEUS was not homogeneous in these 6 patients because there were some occult islands of neoplastic tissue in the thrombus that after administration of ultrasound contrast agent showed as enhancing patterns with respect to the diffuse hypoenhancing of the remaining benign thrombus. Probably in these cases the phenomena of benign thrombosis was superimposed on the initial neoplastic invasion of the portal vein. So CEUS of portal vein thrombi appears as a diagnostic procedure more accurate than "blind" portal FNB in the diagnosis of malignant thrombosis with regard to the possibility of giving a panoramic vision of the thrombus without the sampling-error of "blind" portal FNB. So it is reasonable, when cytology confirmation of malignant thrombosis is needed, that portal FNB can be guided on the result of CEUS in order to reduce false-negative results due to casual sampling.

In conclusion, CEUS of portal thrombus is more accurate than biopsy of thrombus for making the differential diagnosis as to the nature of the thrombus. CEUS of portal thrombus is a reliable diagnostic tool for assessing non-invasively the nature of the PVT. This procedure is usually accurate but presents some sampling errors linked to the 'blind' biopsy of the thrombus.

COMMENTS

Background

About 20% of patients at first access visit to a specialized centre on the care of hepatocellular carcinoma need differential diagnosis between benign portal vein thrombosis (PVT) or malignant thrombosis. Patients who have hepatocellular carcinoma and proven neoplastic vascular thrombus are not candidates for any treatment: in these cases the prevalence of tumor recurrence is nearly 100%.

Research frontiers

The world technique of reference for differentiating benign from malignant PVT is the invasive percutaneous fine needle biopsy (FNB) of the thrombus. Given the obvious clinical utility of a reliable non-invasive technique for the diagnosis of malignant PVT, the authors undertook an investigation to compare Contrast-Enhanced Sonography (CEUS) and portal vein FNB of thrombus for differentiating benign from malignant thrombosis.

Innovations and breakthroughs

For the first time, the authors systematically compare in blinded fashion the validity of portal FNB with respect to non-invasive contrast enhancement of portal thrombus in order to differentiate benign from malignant thrombosis.

Applications

CEUS of portal thrombus is more accurate than biopsy of thrombus for making differential diagnosis of the nature of the thrombus. CEUS of portal thrombus is a reliable diagnostic tool for assessing non-invasively the nature of PVT.

Terminology

CEUS consists of an ultrasound exam which is performed after parenteral administration of an ultrasound contrast. The CEUS in this study consists of sulfurhexafluoride (SF_6) vapor-filled and phospholipid-stabilized microbubbles with a diameter uniformly smaller than 8 μm; these microbubbles circulate in the intravascular space crossing pulmonary and systemic capillary circulation.

Peer review

This paper addresses the value of II Generation CEUS in non-invasive differential diagnosis of benign from malign portal vein thrombosis. The manuscript is interesting.

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BRIEF ARTICLES

Estimating glomerular filtration rate preoperatively for patients undergoing hepatectomy

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Abstract

AIM: To compare creatinine clearance (Ccr) with estimated glomerular filtration rate (eGFR) in preoperative renal function tests in patients undergoing hepatectomy.

METHODS: The records of 197 patients undergoing hepatectomy between August 2006 and August 2008 were studied, and preoperative Ccr, a three-variable equation for eGFR (eGFR3) and a five-variable equation for eGFR (eGFR5) were calculated. Abnormal values were defined as Ccr < 50 mL/min, eGFR3 and eGFR5 < 60 mL/min per 1.73 m^2. The maximum increases in the postoperative serum creatinine (post Cr) level and postoperative rate of increase in the serum Cr level (post Cr rate) were compared.

RESULTS: There were 37 patients (18.8%) with abnormal Ccr, 31 (15.7%) with abnormal eGFR3, and 40 (20.3%) with abnormal eGFR5. Although there were no significant differences in the post Cr rate between patients with normal and abnormal Ccr, eGFR3 and eGFR5 values, the post Cr level was significantly higher in patients with eGFR3 and eGFR5 abnormality than in normal patients (P < 0.0001). Post Cr level tended to be higher in patients with Ccr abnormality (P = 0.0936 and P = 0.0875, respectively).

CONCLUSION: eGFR5 and the simpler eGFR3, rather than Ccr, are recommended as a preoperative renal function test in patients undergoing hepatectomy.

Key words: Estimated glomerular filtration rate; Creatinine clearance test; Hepatectomy; Renal function test

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INTRODUCTION

The outcome of hepatic resection has improved dramatically during the last 20 years, along with improvements in surgical techniques and perioperative management[1]. Operative mortality is now reportedly less than 1% at most institutions in Japan[2]. However, hepatectomy is associated with intraoperative blood loss, and postoperative complications such as liver failure, infection, bile leakage, ascites, and pleural effusion[3]. Uncontrolled ascites, pleural effusion and intraoperative blood loss disturb blood circulation, leading to dysfunction of not only the liver but also the kidney[4]. Therefore, for appropriate patient selection, it is necessary to evaluate preoperative liver and renal function accurately.

Glomerular filtration rate (GFR) is the most important and comprehensive index of renal function. GFR is measured by inulin clearance, but this takes > 2 h, and requires repeat collection of blood and urine every 15 min[5]. GFR is rarely measured in a clinical setting because of its intricacy. On the other hand, creatinine clearance (Ccr) has been measured clinically by a simple

method as a preoperative renal function test[6,7]. Although Ccr yields an approximate value for GFR, it is usually higher than the GFR as a result of secretion of 10%-15% of the creatinine into urine in the uriniferous tubule[8].

In 1999, Levey *et al* first reported a prediction equation known as modification of diet in renal disease (MDRD) for estimation of GFR on the basis of age, sex, race, serum creatinine, albumin and blood urea nitrogen (BUN) level for individuals of caucasian and black ethnicity[8]. MDRD has now been accepted as a standard method for evaluation of renal function in North America and Europe. This was a breakthrough for estimation of GFR because of its simplicity and ease of calculation. As a result of differences in physique between caucasian and black individuals, unique variables for estimating GFR in Japanese subjects have been investigated[9]. For this purpose, in 2008, two new equations for estimated GFR (eGFR) were devised on the basis of multiple regression analysis from inulin clearance data of 763 Japanese patients with chronic kidney disease and healthy controls[10]. These were: the three-variable equation for eGFR (mL/min per 1.73 m^2) = $194Cr^{-1.094} \times Age^{-0.287}$ (× 0.739; if the patient is female); the five-variable equation for eGFR (mL/min per 1.73 m^2) = $142Cr^{-0.923} \times Age^{-0.185} \times Alb^{0.414} \times BUN^{-0.233}$ (× 0.772; if the patient is female).

However, no studies have evaluated the usefulness of eGFR as a preoperative renal function test parameter. If eGFR is superior to Ccr as a preoperative renal function test, then eGFR should replace Ccr because of its simplicity of measurement. In this study, we retrospectively calculated the preoperative three-variable and five-variable equations for eGFR, and compared the results with Ccr, to clarify their superiority as a preoperative renal function test in patients undergoing hepatectomy.

MATERIALS AND METHODS

Patients

At Dokkyo Medical University, a total of 211 hepatic resections were performed for hepatobiliary disease between August 2006 and August 2008. Of these patients, 14 who were on hemodialysis, or for whom the results of preoperative Ccr were not available, were excluded. A total of 197 patients who underwent hepatectomy alone or hepatectomy plus combined surgery such as splenectomy, Hassab's operation, gastrectomy and colectomy for hepatocellular carcinoma (HCC), metastatic liver tumor, biliary malignancy and other benign disease were included in this study. There were 147 men and 50 women, with a mean age of 65.0 ± 10.0 years.

Measurements

Preoperative Ccr was measured by the 24-h method in all patients. The indocyanine green retention rate at 15 min (ICGR15) was also performed before hepatectomy. Serum creatinine, BUN and albumin levels were examined before hepatectomy, and 1, 2, 3, 5, 7, 14, 21 and 28 d after hepatectomy. The preoperative three-variable equation for eGFR (eGFR3) and the five-variable equation for eGFR (eGFR5) were calculated using the new formulas for Japanese patients[10]. The maximum serum creatinine and BUN levels after hepatectomy (post Cr, post BUN) were determined, and the postoperative rate of increase in the serum creatinine level (post Cr rate) was calculated by the following formula using the preoperative serum creatinine level (pre Cr) and post Cr. Post Cr rate (%) = (post Cr - preCr) × 100/post Cr.

Abnormal Ccr was defined as < 50 mL/min according to the New York Heart Association criteria[11,12], and groups with abnormal eGFR3 and eGFR5 were defined as < 60 mL/min per 1.73 m^2 according to the stage of chronic kidney disease[13].

Surgical procedures

Indications for hepatectomy were determined using the criteria of Makuuchi[14]. Portal embolization (PE) before hepatectomy was indicated in patients undergoing major hepatectomy when the estimated liver volume after hepatectomy was not sufficient to tolerate surgery (remnant liver volume < 40%)[15,16]. Transcatheter arterial embolization (TAE) was performed preoperatively in patients with massive HCC in order to occlude arterio-portal shunts. Preoperative biliary drainage was carried out in patients with obstructive jaundice. Liver resection was performed when the serum total bilirubin level was < 2.0 mg/dL. Simultaneous hepatectomy plus splenectomy, or Hassab's operation, were indicated for control of portal hypertension, esophageal and gastric varices, and thrombocytopenia (platelet count < $5.0 \times 10^4/mm^3$) in patients with HCC and liver cirrhosis[17]. The liver parenchyma was transected by the crush method using a Pean forceps or Cavitron Ultrasonic Aspirator while employing the intermittent Pringle maneuver. After resection of the liver tumors and subsequent hemostasis, the cut liver surface was coated with fibrin glue. The abdomen was then closed after placing drains around the cut liver surface. Major hepatectomy was classified as removal of one Couinaud segment or more, and minor hepatectomy as removal of less than one Couinaud segment.

Statistical analysis

Data were expressed as median (range). Non-parametric data were evaluated by χ^2 test and Kruskal-Wallis test between groups showing normal and abnormal values of Ccr, eGFR3 and eGFR5. Parametric data including post Cr and post Cr rate were compared among groups with normal and abnormal Ccr, eGFR3 and eGFR5 values using the Mann-Whitney *U* test. Correlations between Ccr or post Cr and eGFR3 and eGFR5 were analyzed using Pearson's correlation coefficient. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Liver resections were performed for malignant disease in 180 patients and for benign disease in 17 patients. Malignant disease included 117 HCCs, 40 metastatic liver tumors, 16 biliary malignancies, three

Table 1 Indications for hepatectomy in 197 patients

	Overall	Abnormal Ccr group	Abnormal eGFR3 group	Abnormal eGFR5 group
Malignant tumors				
HCC	117	23	15	23
Cholangiocarcinoma	3	1	0	0
Metastatic liver tumor	40	7	7	8
Hilar BDC	12	2	0	1
Gall bladder carcinoma	4	1	2	2
Combined HCC	3	1	1	1
GIST	1	1	1	1
Total	180	36	26	36
Benign diseases				
Hepatolithiasis	2	0	0	0
Hepatic cyst	3	0	2	2
Hemagioma	2	0	1	1
Biliary cyst adenoma	3	1	1	1
Cholecystitis	2	0	1	0
Regenerative nodule	2	0	0	0
Donor	3	0	0	0
Total	17	1	5	4
		$P = 0.9065$	$P = 0.4630$	$P = 0.3889$

HCC: Hepatocellular carcinoma; BDC: Biliary duct carcinoma; GIST: Gastrointestinal stromal tumor.

Table 2 Background characteristics on Ccr

	Normal Ccr group (n = 160)	Abnormal Ccr group (n = 37)	P
Age (yr)	66 (30-82)	71 (46-85)	0.0344
Sex (male:female)	116:44	31:6	0.1552
Height (cm)	160.6 (134.5-179.6)	162.4 (133.9-182.7)	0.4109
Weight (kg)	59.2 (33.0-95.6)	61.6 (44.5-93.0)	0.3543
Hepatitis virus (-:+)	76:84	15:22	0.4441
$ICGR_{15}$ (%)	13 (1-74)	14 (4-49)	0.9085
Preoperative treatment (-:+)	134:26	32:5	0.6804

Hepatitis virus indicates hepatitis B, C, B + C; Preoperative treatment indicates biliary drainage, TAE and portal vein embolization.

cholangiocarcinomas and four other malignancies. Benign lesions included three giant hepatic cysts, three biliary cyst adenomas, three donors of living-related liver transplantation, two cases of hepatolithiasis, two of massive hemangioma, two of cholecystitis, and two cases of regenerative nodules. There were no significant differences in diseases between the groups with normal and abnormal Ccr, eGFR3 and eGFR5 values (Table 1).

Clinical background characteristics of the Ccr, eGFR3 and eGFR5 groups are shown in Tables 2-4, respectively. The median ages of patients with abnormal Ccr and eGFR5 values were significantly greater than those of patients with normal values. There were no significant differences in sex, height, weight, viral infection, $ICGR_{15}$, or frequency of preoperative treatment between the groups with normal and abnormal Ccr, eGFR3 and eGFR5 values.

Thirty-seven patients (18.8%) had abnormal Ccr, 31 (15.7%) had abnormal eGFR3, and 40 (20.3%) had abnormal eGFR5 values. Preoperative serum Cr and BUN levels, Ccr, eGFR3 and eGFR5 in all the patients

Table 3 Background characteristics on eGFR3

	Normal eGFR3 group (n = 160)	Abnormal eGFR3 group (n = 37)	P
Age (yr)	66 (30- 82)	69 (48-85)	0.0887
Gender (male:female)	125:41	22:9	0.6108
Height (cm)	161.0 (133.9-182.7)	160.4 (143.2-173.0)	0.0511
Weight (kg)	59.2 (33.0-93.4)	60.1 (44.6-95.6)	0.1656
Hepatitis virus (-:+)	77:89	14:17	0.9001
$ICGR_{15}$ (%)	13 (3-74)	13 (1-31)	0.9085
Preoperative treatment (-:+)	138:28	28:3	0.3129

eGFR3: Estimating glomerular filtration rate calculated by 3 factors; $ICGR_{15}$: Indocyanine green retention rate at 15 min.

Table 4 Background characteristics on eGFR5

	Normal eGFR5 group (n = 157)	Abnormal eGFR5 group (n = 40)	P
Age (yr)	65 (30-82)	71 (55-85)	0.0003
Sex (male:female)	119:38	28:12	0.4521
Height (cm)	161.5 (133.9-182.7)	158.5 (138.0-173.0)	0.0751
Weight (kg)	59.7 (33.0-95.6)	58.4 (44.0-93.0)	0.5478
Hepatitis virus (-:+)	75:82	16:24	0.3788
$ICGR_{15}$ (%)	13 (3-74)	14 (1-31)	0.6363
Preoperative treatment (-:+)	130:27	36:4	0.2644

with abnormal parameters were significantly worse than those in all the normal patients. Although there were no differences in serum albumin levels between the groups that had normal and abnormal Ccr and eGFR3, the serum albumin level was significantly decreased only in the group with eGFR5 abnormality (Table 5). The correlation between Ccr and eGFR5 was stronger than that between Ccr and eGFR3 (Figure 1).

Surgical details of the patients are shown in Table 6. Seventy-three patients underwent extensive hepatectomy. Among these patients, 28 (14.2%) underwent extended lobectomy, and 19 (9.6%) underwent lobectomy. According to the Couinaud classification, 26 patients (13.2%) underwent bisegmentectomy, and 37 (18.8%) underwent segmentectomy. Eighty-seven patients (44.2%) underwent partial hepatectomy. Although intraoperative blood loss in the patients with eGFR5 abnormality was significantly greater than that of normal patients, there were no significant differences in other surgical background factors, such as operation time, Pringle time, and type of surgical treatment between patients who were normal and abnormal for Ccr, eGFR3 and eGFR5.

Although neither operative nor hospital deaths were recorded, three patients (1.52%) required hemodialysis after hepatectomy because of multiple organ failure (two cases) and enterocolitis. Hepatic failure occurred in two patients. Postoperative results are shown in Table 7. Post Cr and post BUN of patients with eGFR3 and eGFR5 abnormalities were significantly higher than in normal patients, but post Cr and post BUN in patients with Ccr abnormality were not significantly higher than those in

Table 5 Preoperative measurements

	sCr (mg/dL)	BUN (mg/dL)	sAlb (g/dL)	Ccr (mL/min)	eGFR3	eGFR5
Overall (n = 197)	0.73 (0.33-1.74)	13 (5-31)	3.3 (2.1-4.4)	76.8 (1.3-226.1)	77.6 (30.7-196.8)	73.9 (29.8-171.0)
Normal Ccr (n = 160)	0.73 (0.33-1.51)	13 (5-30)	3.3 (2.1-4.4)	86.3 (50.0-226.1)	78.3 (36.7-171.0)	75.1 (36.4-171.0)
	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$
Abnormal Ccr (n = 37)	0.81 (0.35-1.74)	15 (6-31)	3.3 (2.5-4.0)	34.1 (1.3-49.9)	66.6 (30.7-196.8)	64.5 (29.8-144.6)
Normal eGFR3 (n = 166)	0.71 (0.33-1.04)	13 (5-31)	3.3 (2.1-4.4)	83.6 (1.3-226.1)	80.7 (60.2-196.8)	77.0 (45.9-171.0)
	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$
Abnormal eGFR3 (n = 31)	1.05 (0.75-1.74)	17 (9-26)	3.2 (2.1-4.1)	52.4 (11.8-129.1)	52.4 (30.7-59.9)	48.7 (29.8-68.7)
Normal eGFR5 (n = 157)	0.70 (0.33-1.07)	12 (5-22)	3.4 (2.1-4.4)	84.2 (1.3-226.1)	81.0 (58.0-196.8)	78.3 (62.1-171.0)
	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Abnormal eGFR5 (n = 40)	0.97 (0.70-1.74)	18 (10-31)	3.1 (2.1-4.1)	53.6 (11.8-129.1)	55.4 (30.7-68.3)	52.6 (29.8-59.9)

Cr: Creatinine; sCr: Serum creatinine; sAlb: Serum albumine; NS: Not significant.

Table 6 Surgical details

	Operative times (min)	Blood loss (mL)	Pringle time (min)	Hepatectomy (minor:major)	Hepatectomy (alone:plus)
Overall (n = 197)	320 (117-806)	590 (0-12762)	42 (5-176)	124:73	160:37
Normal Ccr (n = 160)	320 (117-721)	573 (0-7240)	43 (5-176)	99:61	132:28
	NS	NS	NS	NS	NS
Abnormal Ccr (n = 37)	321 (180-806)	618 (114-12762)	35 (9-98)	25:12	28:9
Normal eGFR3 (n = 166)	323 (117-721)	553 (0-7240)	42 (5-176)	103:63	136:30
	NS	NS	NS	NS	NS
Abnormal eGFR3 (n = 31)	297 (168-806)	680 (126-12762)	46 (12-87)	21:10	24:7
Normal eGFR5 (n = 157)	325 (117-721)	551 (0-7240)	42 (5-176)	97:60	128:29
	NS	$P < 0.05$	NS	NS	NS
Abnormal eGFR5 (n = 40)	296 (142-806)	694 (126-12762)	44 (11-98)	27:13	32:8

Minor: < 1 segmentectomy; Major: ⩾ 1 segmentectomy; Alone: Only hepatectomy; Plus: Hepatectomy with combined surgery.

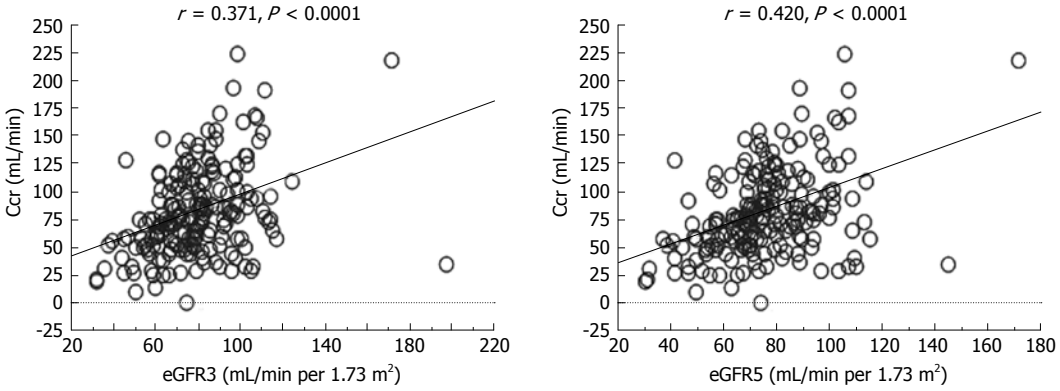

Figure 1 Relationship of measured Ccr to eGFR.

in normal patients.

Figure 2 shows the correlation between Ccr, eGFR3 and eGFR5 and post Cr. Although a weak correlation between post Cr and Ccr was observed, there were significant correlations between post Cr and eGFR5, and the correlation between post Cr and eGFR5 was higher than that between post Cr and eGFR3. Post Cr rates of patients with Ccr, eGFR3 and eGFR5 abnormality were not significant (Table 7).

DISCUSSION

As a preoperative renal function test, it is ideal to measure GFR by inulin clearance, but this is not practical in a clinical setting. Although the easiest renal function test to perform is measurement of serum creatinine level, use of this parameter alone is not recommended because it is affected by various factors such as muscle mass, sex, age, diet, and renal tubule function[9,13]. Therefore, Ccr has been measured routinely in patients undergoing major surgery for a long time. Determination of Ccr requires timed urine collection and blood sampling. Twenty-four-hour urine collection is especially inconvenient for patients with neurogenic bladder or the elderly. On the other hand, eGFR3 and eGFR5 require only a single blood sample, and can be estimated on the basis of age, sex, serum creatinine, BUN and albumin without the need for urine collection. If eGFR3

Table 7 Postoperative measurements

	Post Cr (mg/dL)	Post BUN (mg/dL)	Post Cr rate (%)
Overall (n = 197)	0.87 (0.43-8.43)	18 (7-97)	11.3 (0-88.1)
Normal Ccr (n = 160)	0.85 (0.43-5.71)	17 (7-81)	11.0 (0-88.1)
	P = 0.0936	P = 0.0875	P = 0.8253
Abnormal Ccr (n = 37)	0.95 (0.50-8.43)	20 (10-97)	12.3 (0-79.4)
Normal eGFR3 (n = 166)	0.83 (0.43-5.71)	17 (7-81)	11.4 (0-88.1)
	P < 0.0001	P = 0.0033	P = 0.4575
Abnormal eGFR3 (n = 31)	1.17 (0.70-8.43)	21 (11-97)	9.0 (0-79.4)
Normal eGFR5 (n = 157)	0.81 (0.43-5.71)	16 (7-81)	11.3 (0-88.1)
	P < 0.0001	P < 0.0001	P = 0.8950
Abnormal eGFR5 (n = 40)	1.14 (0.70-8.43)	23 (11-97)	11.5 (0-79.4)

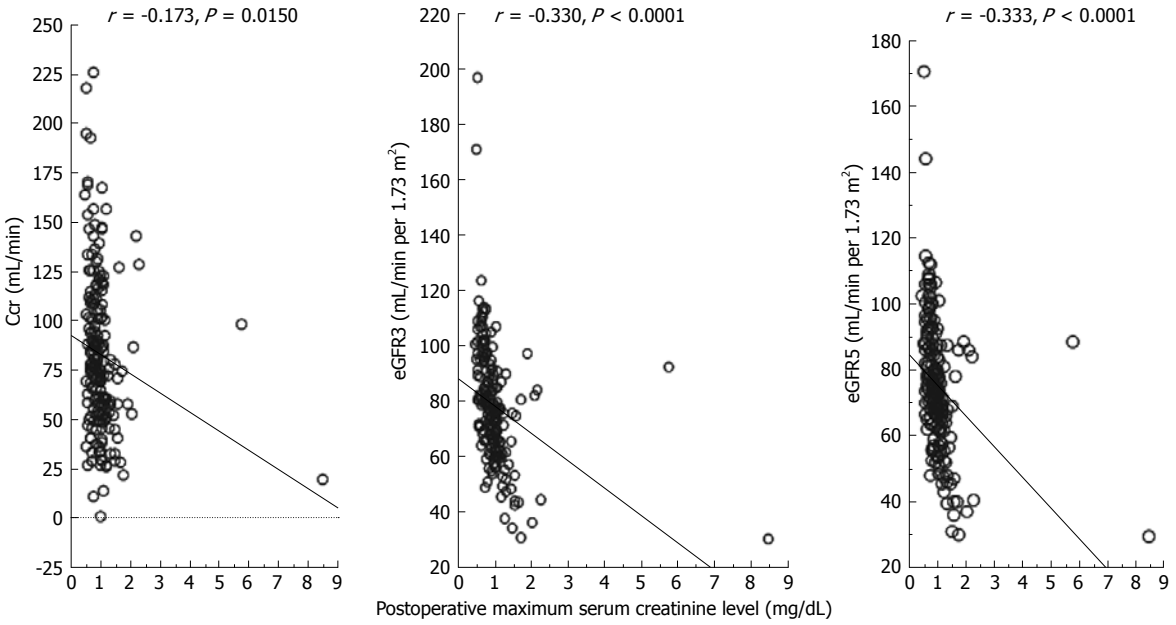

Figure 2 Relationship of the maximum increase in post Cr level to measured Ccr and eGFR.

and eGFR5 are superior to Ccr for preoperative renal function testing, there would be certain advantages in terms of clinical effort and cost.

The results of this study demonstrated that 24-h urine collection for measurement of Ccr no longer appears necessary on a routine basis for estimation of preoperative renal function. In fact, there were no significant differences in the post Cr or BUN level after hepatectomy between patients who had normal and abnormal preoperative Ccr values (Table 7). Post Cr and BUN levels in patients with eGFR3 and eGFR5 abnormalities were significantly higher than those in normal patients. In addition, the correlations between post Cr and eGFR3, and eGFR5 were significant, but that between post Cr and Ccr was not significant (Figure 2). These results indicate that eGFR3 and eGFR5 are superior to Ccr for predicting post renal dysfunction.

In this study, the post Cr rate after hepatectomy was also evaluated in patients who had normal and abnormal Ccr, eGFR3 and eGFR5 values, and no significant differences were evident (Table 7). We have already reported that hepatectomy can be performed safely without rapid and progressive deterioration of renal function in patients with non-uremic renal failure (Ccr of > 20 but < 50 mL/min)[6]. The factors affecting the post Cr rate after hepatectomy are preoperative liver function ($ICGR_{15}$ > 20%), intraoperative blood loss and operation time, and not preoperative renal dysfunction (data not shown).

In this study, eGFR5 differentiated 40 patients with preoperative renal dysfunction among 197 patients more sensitively than Ccr or eGFR3. There was a stronger positive correlation between Ccr and eGFR5 than between Ccr and eGFR3 (Figure 1). Although the equation for eGFR5 is a little complex, eGFR5 is more suitable than eGFR3 for patients undergoing hepatectomy. Since serum albumin level is one of the factors that reflects liver preservation, patients with HCC and liver cirrhosis frequently have lower levels of serum albumin. In fact, in this study, preoperative serum albumin levels ranged from 2.1 to 4.4 g/dL, with a median value of 3.3 g/dL. Thus, eGFR5 appears to be a more acceptable parameter for accurate preoperative evaluation of renal function in hepatectomy patients presenting a wide range of serum albumin levels.

To the best of our knowledge, this is the first retrospective study to have compared Ccr and eGFR as a preoperative renal function test in patients undergoing

hepatectomy. Since equations for eGFR in individuals of caucasian, black and Japanese ethnicity have been established, eGFR is now almost universally available. We suggest that eGFR3 and eGFR5 are useful as preoperative renal function parameters in patients undergoing hepatectomy worldwide.

In conclusion, we recommend eGFR5 using serum albumin level as a preoperative renal function test in patients undergoing hepatectomy. Ccr is no longer recommended as a first-choice preoperative renal function test.

COMMENTS

Background

Although creatinine clearance (Ccr) has been measured clinically by a simple method as a preoperative renal function test, Ccr is not strictly equal to glomerular filtration rate (GFR). Recently, an equation for estimated GFR (eGFR) for Japanese individuals has been postulated. It has been accepted that eGFR is equal to measured GFR in chronic kidney disease. However, there have been no previous studies regarding the reliability of eGFR as a preoperative renal function test.

Research frontiers

If eGFR is superior to, or equal to Ccr as a preoperative renal function test, eGFR should replace Ccr because of its simplicity of measurement. The authors retrospectively compared Ccr and eGFR as a preoperative renal function test in patients undergoing hepatectomy.

Innovations and breakthroughs

eGFR is useful as preoperative renal function parameters in patients undergoing hepatectomy. Ccr is no longer recommended as a first-choice preoperative renal function test.

Applications

Although Ccr has been used as preoperative renal function test, eGFR should replace Ccr as a routine preoperative renal function test in various surgical fields.

Terminology

eGFR is estimated GFR which is calculated from age, sex, serum creatinine value (eGFR3), or adding serum albumin concentration and BUN value (eGFR5).

Peer review

This is a well-written paper on normal and sensitive parameters of renal function, i.e. eGFR3 and eGFR5 as predictors of renal function after hepatectomy. These parameters seem easy to determine, accurate and well-associated with the stage of kidney disease. The study seems well-designed and performed, original in concept and statistically valid.

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BRIEF ARTICLES

Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma

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Abstract

AIM: To verify that CD markers are available for detecting cancer stem cell populations and to evaluate their clinical significance in colon cancer.

METHODS: Immunohistochemistry for CD133, CD24 and CD44 was performed on the tissue microarray of 523 colorectal adenocarcinomas. Medical records were reviewed and clinicopathological analysis was performed.

RESULTS: In colorectal adenocarcinoma, 128 of 523 cases (24.5%) were positive and 395 cases (75.5%) were negative for CD133 expression. Two hundred and sixty-four of 523 cases (50.5%) were positive and 259 cases (49.5%) were negative for CD24 expression. Five hundred and two of 523 cases (96%) were negative and 21 cases (4%) were positive for CD44 expression. Upon clinicopathological analysis, CD133 expression was present more in male patients (P = 0.002) and in advanced T stage cancer (P = 0.024). Correlation between CD24 expression and clinicopathological factors was seen in the degree of differentiation (P = 0.006). Correlation between CD44 expression and clinicopathological factors was seen in the tumor size (P = 0.001). Survival was not significantly related to CD133, CD24 and CD44 expression.

CONCLUSION: CD markers were related to invasiveness and differentiation of colorectal adenocarcinoma. However, CD expression was not closely related to survival.

Key words: CD133; CD24; CD44; Colon cancer stem cells; Colorectal adenocarcinoma

Peer reviewer: Finlay A Macrae, MD, Professor, Royal Melbourne Hospital, Po Box 2010, Victoria 3050, Australia

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INTRODUCTION

Colorectal adenocarcinoma is the second most common type of cancer and a major cause of cancer-related morbidity and mortality in the Western world[1]. The incidence of colorectal cancer has increased from 5.8% in 1980 to 10.3% in 2000 in South Korea, in part because of Westernization of the diet[2].

Countless treatment protocols, including chemotherapy and radiation, have been applied to colorectal cancer and a number of studies have identified conventional prognostic factors[3]. However, a complete cure of colorectal cancer has not been accomplished despite numerous efforts. Recently, the prospective identification of colon cancer stem cells

has received major attention because of their potential for colon cancer treatment[4,5]. Current colon cancer treatment modalities target proliferating cells, but colon cancer stem cells are thought to be slowly cycling cells; therefore, they may escape present targeted interventions because they are not actively proliferating. This may be one of the most important reasons behind colon cancer treatment failure and recurrence. It is important to validate *in vitro/in vivo* colon cancer stem cell findings in clinical samples. This will be a critical step toward the development of effective targeted colon cancer treatment, but thus far, no data are available on the clinical implications of the suggested colon cancer stem cells in clinical samples.

Recently, several CD markers have been identified as solid cancer stem cell markers. CD133, also known as PROML1 or prominin, is a stem cell surface antigen that has been recently identified as a potential cancer stem cell marker in brain, colon and prostate cancer[4-7]. CD44, also known as homing cell adhesion molecule, is a cell surface glycoprotein expressed on lymphocytes, monocytes and granulocytes, which has been identified as a stem cell marker in breast and head and neck cancer[8,9]. CD24, a cell surface marker, is a single chain sialoglycoprotein with a molecular mass of 42 kDa. CD24- and CD44-expressing pancreatic cancer cells show cancer stem cell characteristics[10]. Here, we report the identification of CD133-, CD24- and CD44-positive tumor cells in colon tumor sections by an immunohistochemistry-based technique, and discuss the findings in conjunction with clinicopathological data.

MATERIALS AND METHODS

Patients and specimens

This retrospective study consisted of a consecutive series of 523 colorectal adenocarcinomas with complete histopathological data available. Patients were diagnosed and treated at the Hanyang University Hospital, Seoul, Korea, from January 1991 to August 2001. There were 295 male and 228 female patients, with ages ranging from 17 to 87 years (mean, 59.0 years). The adenocarcinomas were located in the cecum (n = 18), ascending colon (n = 77), hepatic flexure (n = 12), transverse colon (n = 26), splenic flexure (n = 4), descending colon (n = 24), sigmoid colon (n =112), and rectum (n = 250). Their sizes ranged from 0.3 to 15 cm (mean, 5.7 cm).

All tissue samples were formalin-fixed and paraffin-embedded. Hematoxylin and eosin (HE)-stained slides, pathological reports, and other medical records were reviewed to confirm the diagnosis and clinicopathological parameters, including age, gender, tumor location, tumor size, depth of invasion, lymph node metastasis, distant metastasis, American Joint Committee on Cancer (AJCC) stage, Dukes' stage, degree of differentiation, lymphovascular invasion and patient survival.

Tissue microarray (TMA) construction

The most representative area was carefully selected and marked on an H&E-stained slide. The TMA was assembled using a tissue-array instrument (AccuMac arrayer; ISU ABXIS Co. Ltd., Seoul, Korea) that consisted of thin-walled stainless steel punches and stylets used to empty and transfer the needle content. The assembly was held in an X-Y position guide equipped with semiautomatic micrometers, with a 1-mm increment between individual samples and a 3-mm punch depth stop device. Briefly, the instrument was used to create holes in a recipient block with defined array cores. A solid stylet, which closely fits the needle, was used to transfer the tissue cores into the recipient block. Taking into account the limitations of the representative areas of the tumor, we used triplicate 1-mm diameter tissue cores from each donor block.

HE and immunohistochemical staining

Multiple 4-μm sections were cut with a Leica microtome. Sections were transferred to adhesive-coated slides. One section was routinely deparaffinized with standard xylene and hydrated through graded ethanol in water, stained with HE, and covered with a coverslip. For immunohistochemical staining, the TMA slides were dewaxed by heating at 55℃ for 30 min and by three 5-min washes with xylene. Tissues were rehydrated by a series of 5-min washes in 100%, 90% and 70% ethanol and phosphate buffered saline (PBS). Antigen retrieval was performed by microwaving the samples for 4 min 20 s at full power in 250 mL 10 mmol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 20 min. The primary polyclonal rabbit anti-CD133 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:200 using goat serum and incubated at room temperature for 1 h. The primary monoclonal mouse anti-CD24 antibody (Santa Cruz Biotechnology) was diluted 1:50 and the primary monoclonal mouse anti-CD44s antibody (Neomarkers, CA, USA) was diluted 1:100. After three 2-min washes with PBS, the sections were incubated with a biotinylated goat secondary antibody for 30 min (DAKO, Carpinteria, CA, USA). After three 2-min washes with PBS, horseradish peroxidase-streptoavidin (DAKO) was added to the section for 30 min, followed by another three 2-min washes with PBS. The samples were developed with 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) for 1 min and counterstained with Mayer's hematoxylin. The slides were dehydrated following a standard procedure and sealed with coverslips. We used the glioblastoma tissue as a positive control of CD133, the tonsillar lymphoid tissue as a positive control of CD24, and the tonsillar mucosal epithelial tissue as a positive control of CD44. Negative controls were performed by omitting CD133, CD24 and CD44 antibodies during the primary antibody incubation. The representative sections of CD133, CD24 and CD44 immunostaining are shown in Figure 1.

Interpretation of CD133, CD24 and CD44 expression

CD133, CD24 and CD44 expression was evaluated

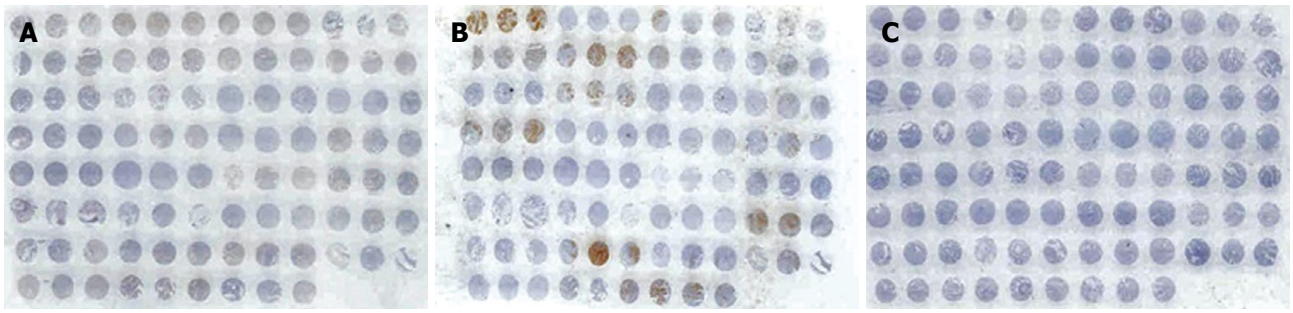

Figure 1 Representative photograph of the TMA slides with immunohistochemical staining. A: CD133; B: CD24; C: CD44.

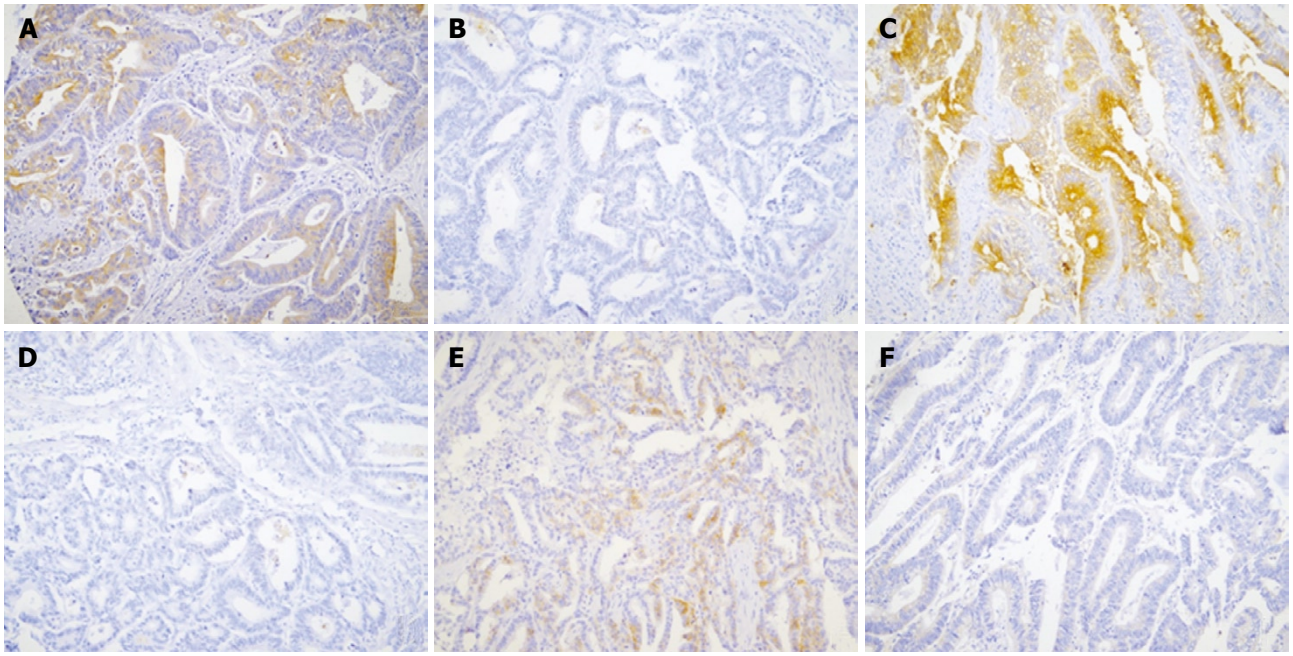

Figure 2 Representative photographs of CD marker expression in colorectal adenocarcinoma. Positive staining (A) and negative staining (B) for CD133. Positive staining (C) and negative staining (D) for CD24. Positive staining (E) and negative staining (F) for CD44.

semi-quantitatively by two independent pathologists (Paik SS and Song YS), in a blinded fashion without knowledge of clinical and pathological information. The sections were scanned at high magnification to assess the positivity of staining in tumor cells. We regarded the staining as positive in cases with cytoplasmic positivity. In cases of discrepant assessments, slides were reinvestigated by both pathologists under a multi-head microscope and an agreement was obtained.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0 software (SPSS, Chicago, IL, USA). The χ^2 test was used to examine the association between CD133, CD24 and CD44 expression and various clinicopathological characteristics, including age, gender, tumor location, tumor size, TNM category, AJCC stage, Dukes' stage, degree of differentiation, and lymphovascular invasion. The Kaplan-Meier method was used to calculate overall survival curves. Univariate survival analysis with the log-rank test was used to compare the difference between the survival rates of the patients' subgroups. Multivariate survival analysis with Cox's proportional hazard regression model was used to evaluate the independent prognostic factors. A difference of $P < 0.05$ between groups was considered significant.

RESULTS

Pattern of CD marker expression in colorectal adenocarcinoma

CD marker expression was evaluated in colorectal adenocarcinoma. One hundred and twenty-eight of 523 cases (24.5%) were positive and 395 cases (75.5%) were negative for CD133 expression (Figure 2A and B). Two hundred and sixty-four of 523 cases (50.5%) were positive and 259 cases (49.5%) were negative for CD24 expression (Figure 2C and D). However, 21 of 523 cases (4%) were positive and 502 cases (96%) were negative for CD44 expression (Figure 2E and F).

Correlation of CD marker expression and clinicopathological parameters in colorectal adenocarcinoma

Upon clinicopathological analysis, CD133 expression

Table 1 Correlation between CD133 and CD24 expression and clinicopathological factors in colorectal cancer (*n* = 523)

	n	Expression of CD133		*P* value	Expression of CD24		*P* value
		Negative (*n* = 395)	Positive (*n* = 128)	(χ^2-test)	Negative (*n* = 259)	Positive (*n* = 264)	(χ^2-test)
Age (yr)				0.431			0.999
< 59	261	201	60		129	132	
⩾ 59	262	194	68		130	132	
Gender				0.002			0.261
Male	295	208	87		140	155	
Female	228	187	41		120	108	
Tumor location				0.735			0.315
Right colon	133	99	34		71	62	
Left colon	390	296	94		189	201	
Tumor size				0.436			0.658
< 5.5 cm	254	188	66		124	130	
⩾ 5.5 cm	269	207	62		136	133	
T category				0.024[1]			0.219
Tis	12	12	0		8	4	
T1	9	8	1		4	5	
T2	37	29	8		20	17	
T3	452	337	115		223	229	
T4	13	9	4		5	8	
N category				0.890			0.525
N0	234	178	56		113	121	
N1	132	96	36		65	67	
N2	157	121	36		81	76	
M category				0.555			0.482
M0	502	378	124		248	254	
M1	21	17	4		12	9	
AJCC stage				0.259			0.998
0	12	12	0		8	4	
I	34	29	5		18	16	
ⅡA, ⅡB	185	134	51		85	100	
ⅢA, ⅢB, ⅢC	271	203	68		137	134	
Ⅳ	21	17	4		12	9	
Dukes' stage				0.560			0.515
A	17	16	1		10	7	
B1, B2	210	157	53		98	112	
C1, C2	275	205	70		139	136	
D	21	17	4		12	9	
Degree of differentiation				0.084			0.006[1]
Well	23	16	7		10	13	
Moderate	393	300	93		183	210	
Poorly	100	74	26		61	39	
Undifferentiated	7	5	2		5	2	
Lymphatic invasion				0.848			0.772
Absent	225	169	56		110	115	
Present	298	226	72		150	148	
Vascular invasion				0.740			0.508
Absent	513	387	126		254	259	
Present	10	8	2		6	4	

[1]χ^2 test for linear trend. AJCC: American Joint Committee on Cancer; CD: Cluster of differentiation.

was present more in male patients (P = 0.002) and in advanced T stage cancer (P = 0.024) (Table 1). Correlation between CD24 expression and clinicopathological factors was seen in the degree of differentiation (P = 0.006) (Table 1). Correlation between CD44 expression and clinicopathological factors was seen for the tumor size (P = 0.001) (data not shown).

Correlation of CD marker expression and patient overall survival

We examined the effect of CD marker expression on clinicopathological prognostic factors in colorectal adenocarcinoma. A significant prognostic influence of age, histological grade, AJCC stage, lymphatic invasion and vascular invasion on overall survival was found by univariate and/or multivariate analyses. However, no impact of CD133, CD24 and CD44 expression on overall survival was observed in univariate and multivariate survival analyses. Kaplan-Meier survival curves and log-rank tests showed no significant correlation between patient survival and CD133, CD24 and CD44 expression (P = 0.774, P = 0.775 and P = 0.636, respectively) (Figure 3).

DISCUSSION

Cancer stem cells have recently been proposed to

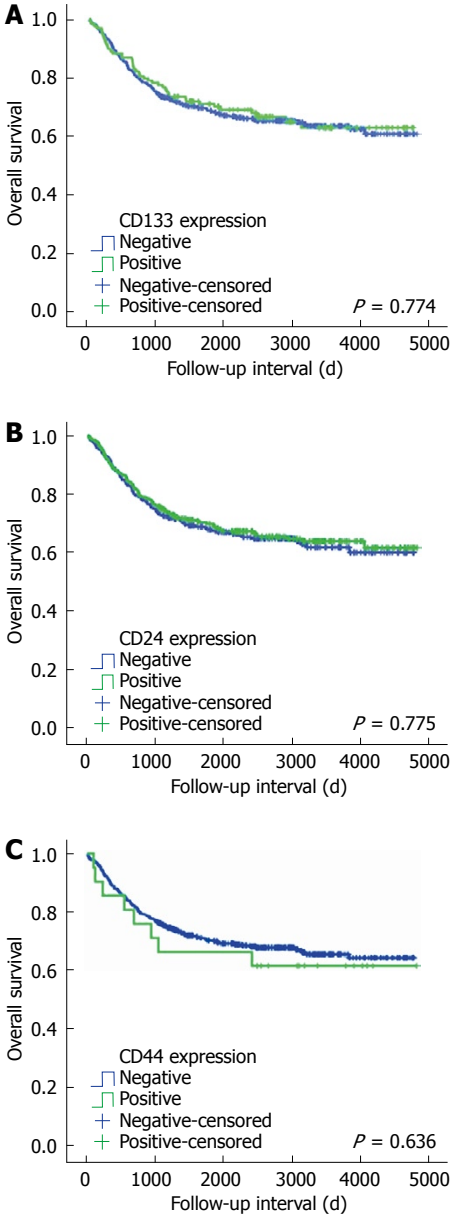

Figure 3 Cumulative survivals according to CD133 (*P* = 0.774) (A), CD24 (*P* = 0.775) (B) and CD44 (*P* = 0.636) (C) expression in colorectal cancer patients (Kaplan-Meier method).

be the cancer initiating cells that are responsible for tumorigenesis and for contributing to cancer resistance in leukemia[11]. Compared to leukemia, evidence for the existence of cancer stem cells in solid tumors has been more difficult to obtain for several reasons. Cells within solid tumors are less accessible, and functional assays suitable for detecting and quantifying normal stem cells from many organs have not yet been developed, and the cell surface markers required to isolate such cells have not been identified fully. However, there have been some impressive studies in this area recently. Advances have been made in identifying and enriching cancer stem cells in several solid tumors, including breast, brain and colon cancers[4-6,8-10].

Lapidot *et al*[12] have shown that leukemia-initiating stem cells present in the peripheral blood of acute myelogenous leukemia (AML) patients can induce AML when transplanted into severe combined immunodeficient mice. In 2003, Al-Hajj *et al*[8] isolated human breast cancer stem cells that can cause breast cancer in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice through serial transplantations, which suggests a capacity for self-renewal. The following year, Singh *et al*[6] have found evidence of stem cell involvement in brain cancer. Recently, O'Brien *et al*[4] have demonstrated that CD133-positive colon-cancer-initiating cells in the human colon cancer specimen generate tumors in the renal capsule of pre-irradiated NOD/SCID mice. More recently, cells have been isolated from human prostate cancer patients, which can produce serially transplantable prostate tumors in NOD/SCID mice[10]. Even though definitive cancer stem cell markers have not been found in all the previously mentioned studies, these studies have revealed that only a small subset of cells in different tumor types is capable of tumor formation and several candidate stem cell markers have been evaluated. While CD133, CD24 and CD44 have been tested as cancer stem cell markers for serial transplantation studies in various cancers, their prognostic value has not been elucidated clearly[4-10].

CD133, which is one of the most important cancer stem cell markers[4-7], was stained fairly well in 24.5% of our colon cancer patients. In terms of clinicopathological parameters, CD133 expression was related to gender and T stage. The present study revealed that male gender was positively related to CD133 expression. T0 and T1 colon cancers showed lower incidence of CD133 protein expression compared to advanced colon cancer.

CD24, another important cancer stem cell marker, was expressed in 50.5% of the colon cancer patients. Correlation between CD24 expression and clinicopathological factors was seen in degree of differentiation. CD24 consists of a small protein core comprising 27 amino acids, which is extensively glycosylated and is bound to the membrane via a phosphatidylinositol anchor[13-15]. Several reports have shown that CD24 can be expressed on several solid tumors such as small cell lung cancer, neuroblastoma, rhabdomyosarcoma and renal cell cancer[16,17]. Lim *et al*[18] have reported that CD24 expression is related to lymph node metastasis in colon cancer. Weichert *et al*[19] have shown that cytoplasmic CD24 expression in colorectal cancer is independently correlated with shortened patient survival. We have not seen any positive correlation between CD24 expression and nodal status and patient survival.

CD44 is an unique adhesion molecule and several studies have revealed that CD44 is overexpressed at the mRNA and protein levels in colon cancer[20,21]. In our study, large tumors bigger than 5.5 cm showed more frequent CD44 expression, which indicated that CD44 expression was related to clinical tumor burden.

Our results in human colon cancer specimens showed various expression patterns of CD markers. This is believed to be the first trial to verify the

relationship between well-known prognostic factors of colon cancer and conventional cancer stem cell markers. Tumor invasiveness and differentiation were identified as clinicopathological factors related to cancer stem cell markers, especially CD133 and CD24.

For further study, other colon stem cell markers which are related to patient survival should be found for clinical application of colon cancer stem cells. Several studies have indicated that pleuripotency-related factors, including Oct3/4, are related to cancer development[22-24]. Beside CD markers, other cancer stem cell markers may help distinguish cancer stem cells from cancer cells.

In summary, we have presented some evidence that several conventional clinical factors were related to cancer stem cell markers in colorectal adenocarcinoma. However, CD133, CD24 and CD44 expression did not show a close relationship with the survival outcome of colorectal adenocarcinoma. These results warrant further careful and well-designed studies of colon cancer stem cells as markers for clinical application.

COMMENTS

Background

Colorectal adenocarcinoma is the second most common type of cancer and a major cause of cancer-related morbidity and mortality in the Western world. Countless treatment protocols including chemotherapy and radiation have been applied to colorectal cancer treatment and a number of studies have identified conventional prognostic factors. Recently, the prospective identification of colon cancer stem cells has received major attention because of their potential for colon cancer treatment.

Research frontiers

This study focused on the identification of CD133-, CD24- and CD44-positive tumor cells in colon tumor sections by an immunohistochemistry-based technique, and the findings are discussed in conjunction with clinicopathological data.

Innovations and breakthroughs

Results in human colon cancer specimens showed various expression patterns of CD markers. This is believed to be the first trial for verifying the relationship between well-known prognostic factors of colon cancer and cancer stem cell markers. Tumor invasiveness and differentiation were identified as clinicopathological factors related to cancer stem cell markers.

Applications

The authors have presented some evidence that several conventional clinical factors were related to cancer stem cell markers in colorectal adenocarcinoma. However, CD133, CD24 and CD44 expression did not show a close relationship with survival. These results warrant further careful and well-designed studies of colon cancer stem cells as markers for clinical application.

Terminology

CD133, also known as PROML1 or prominin, is a stem cell surface antigen that has been identified recently as a potential cancer stem cell marker in brain, colon and prostate cancer. CD44, also known as homing cell adhesion molecule, is a cell surface glycoprotein expressed on lymphocytes, monocytes and granulocytes, which has been identified as a stem cell marker in breast and head and neck cancer. CD24, a cell surface marker, is a single chain sialoglycoprotein with a molecular mass of 42 kDa.

Peer review

The authors have presented some evidence that several conventional clinical factors were related to cancer stem cell markers in colorectal adenocarcinoma. This study is modestly reported with respect to a careful and large study. The approach needs to be encouraged, despite the relatively negative nature of the study with respect to the utility of the markers as a prognostic markers.

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How good is cola for dissolution of gastric phytobezoars?

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Abstract

AIM: To evaluate the efficacy of cola treatment for gastric phytobezoars, including diospyrobezoars.

METHODS: A total of 17 patients (range: 48 to 78 years) with symptomatic gastric phytobezoars treated with cola and adjuvant endoscopic therapy were reviewed. Three liters of cola lavage (10 cases) or drink (7 cases) were initially used, and then endoscopic fragmentation was done for the remnant bezoars by using a lithotripsy basket or a polypectomy snare. The overall success of dissolving a gastric phytobezoars with using three liters of cola and the clinical and endoscopic findings were compared retrospectively between four cases of complete dissolution by using only cola and 13 cases of partial dissolution with cola.

RESULTS: After 3 L of cola lavage or drinking, a complete dissolution of bezoars was achieved in four patients (23.5%), while 13 cases (76.5%) were only partially dissolved. Phytobezoars (4 of 6 cases) were observed more frequently than diospyrobezoars (0 of 11) in the group that underwent complete dissolution (P = 0.006). Gender, symptom duration, size of bezoar and method of cola administration were not significantly different between the two groups. Twelve of 13 patients with residual bezoars were completely treated with a combination of cola and endoscopic fragmentation.

CONCLUSION: The rate of complete dissolution with three liters of cola was 23.5%, but no case of diospyrobezoar was completely dissolved using this method. However, pretreatment with cola may be helpful and facilitate endoscopic fragmentation of gastric phytobezoars.

Key words: Gastric phytobezoars; Diospyrobezoars; Cola; Dissolution; Clinical efficacy

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Lee BJ, Park JJ, Chun HJ, Kim JH, Yeon JE, Jeen YT, Kim JS, Byun KS, Lee SW, Choi JH, Kim CD, Ryu HS, Bak YT. How good is cola for dissolution of gastric phytobezoars? *World J Gastroenterol* 2009; 15(18): 2265-2269 Available from: URL: http://www.wjgnet.com/1007-9327/15/2265.asp DOI: http://dx.doi.org/10.3748/wjg.15.2265

INTRODUCTION

Bezoars are hard masses or concretions of indigestible food, vegetable fiber or hair that are found in the gastrointestinal (GI) tract, usually in the stomach. Although the incidence of bezoars is unknown, the reported incidence is about 0.4%[1]. Bezoars are usually classified into four types according to their composition: phytobezoars, trichobezoars, medication bezoars and lactobezoars. Dissolution therapy with proteolytic or cellulase enzymes[2], endoscopic fragmentation or aspiration and surgery have been proposed as the treatment options for bezoars, and these treatments have a wide range of efficacy[3]. Ingestion of persimmons is considered the most common cause of phytobezoars in some countries[4,5]. Because of their hard consistency, endoscopic therapy with fragmentation or enzymatic dissolution is challenging and sometimes mechanical fragmentation cannot be accomplished.

Recently, dissolution bezoars with cola has been described to be one of the effective treatment options for the treatment of gastric phytobezoars. However,

Table 1 Basal characteristics of 17 consecutive patients

Case no.	Gender	Age	Symptoms	Duration (d)	Comorbidity	Type of bezoar	Endoscopic findings	Size of bezoar
CD group								
1	F	69	Indigestion	21	None	Phytobezoar	GU	Above 50%
2	F	62	Epigastric soreness	21	DM	Phytobezoar	GU	Above 50%
3	F	49	Indigestion, epigastric soreness	14	DM	Phytobezoar	Pyloric stenosis	Below 50%
4	F	51	Indigestion	20	DM	Phytobezoar	GU	Below 50%
PD group								
5	M	48	Epigastric soreness	8	None	Phytobezoar	DU scar with stenosis	Below 50%
6	F	57	Indigestion	21	None	Diospyrobezoar	Pyloric stenosis	Above 50%
7	M	71	Indigestion	90	None	Diospyrobezoar	Pyloric stenosis	Below 50%
8	F	65	Epigastric soreness	10	HTN	Diospyrobezoar	Pyloric stenosis	Above 50%
9	F	61	Indigestion	14	DM	Diospyrobezoar	Pyloric stenosis	Below 50%
10	F	57	Indigestion	21	None	Diospyrobezoar	Pyloric stenosis	Below 50%
11	F	67	Epigastric soreness	60	DM	Diospyrobezoar	GU	Below 50%
12	M	63	Epigastric soreness	21	HTN	Phytobezoar	Pyloric stenosis	Above 50%
13	M	78	Indigestion	30	DM	Diospyrobezoar	GU	Above 50%
14	F	75	Epigastric soreness	90	DM	Diospyrobezoar	None	Below 50%
15	F	54	None	7	HTN	Diospyrobezoar	GU	Below 50%
16	F	61	Nausea and vomiting	7	None	Diospyrobezoar	GU	Below 50%
17	M	63	Epigastric soreness	30	None	Diospyrobezoar	Pyloric stenosis	Above 50%

GU: Gastric ulcer; DM: Diabetes mellitus; CD: Complete dissolution; PD: Partial dissolution.

only commentaries on individual cases or small number of cases have been reported on the cola dissolution of phytobezoars[6-12]. To elucidate the efficacy of cola for dissolving phytobezoars, further evaluations with large number cases are needed. Here, we report the clinical results of 17 consecutive patients with gastric phytobezoars, including diospyrobezoars, who were initially treated by three liters of cola gastric lavage or drink.

MATERIALS AND METHODS

The medical records of 17 patients with gastric phytobezoars treated by cola gastric lavage at Korea University Hospital, Seoul, between 2002 and 2008 were retrospectively reviewed. Diagnosis and classification of gastric bezoars were based upon clinical and endoscopic findings. Diospyrobezoar was diagnosed by history of eating persimmons and the endoscopic typical characteristics such as cemented seeds of persimmon or hard consistency or darkish-brown color. The size of each bezoar was grossly estimated as occupied percentage of the lumen of the stomach. The patients' median age was 63 years (range: 48-78). Seven patients had a long-standing history of diabetes mellitus (DM), four had a history of arterial hypertension (AHT) and the other patients had no significant past or concurrent diseases. On the initial endoscopic evaluation, 11 cases had darkish-brown colored diospyrobezoar and 6 cases had bright brown or yellow colored phytobezoars (Table 1). After diagnosing the bezoar, two nasogastric tubes (16 F) were placed for performing gastric cola lavage. One tube was used for the continuous administration of cola, and the other for natural drainage. A lavage with three liters of cola was performed over 12 h. All the procedures were done after obtaining informed consent from all the patients. Among the 17 patients, 8 patients drank the three liters of cola as they had refused to have the nasogastric tube inserted (Table 2). The nine patients with nasogastric tubes were kept in the recumbent position to prevent aspiration. The patients with DM had blood glucose monitoring by a glucometer. One day after the lavage with three liters of cola (or after drinking the same amount of cola), second-look endoscopic examinations were performed for assessing the bezoar. The status of the bezoars was then described, i.e. whether or not the bezoar remained in place, and whether it had changed its size and consistency. When the bezoar had disappeared, we considered this as complete dissolution. When it had a decreased size and a softened consistency by palpation with the biopsy forcep, then the response was considered as partial dissolution. If no change of the size and consistency was observed, then this was considered as lack of response. As the result of the initial administration of cola, patients were divided into two groups, i.e with either complete dissolution or partial dissolution. The clinical and endoscopic features and the method of cola administration were then retrospectively compared between two groups.

The endoscope we used was an Olympus GIF Q 260 or a H 260 (Olympus, Tokyo, Japan). If a residual bezoar was observed, then endoscopic fragmentation was done using a lithotripsy basket (GML-90-26-180, Medi-Globe, Achenmuhle, Germany) or a polypectomy snare (SD-5L-1, Olympus, Japan) (Figure 1A and B). Finally, fragmented bezoars were crushed and retrieved with a biopsy forcep or a pentagon grasping forcep (Figure 1C and D). After the procedures, all the patients were advised to drink an additional 3 L of cola. Re-examination endoscopy was done one day after the procedures. All the endoscopic procedures were performed by three expert endoscopists.

Fisher exact test and the Mann-whitney *U* test were used for the comparison of clinical, endoscopic, and method of administration of cola. $P < 0.05$ were regarded as statistically significant.

RESULTS

After lavage with (or drinking) 3 L of cola, a complete

Table 2 Results of cola administration and endoscopic intervention

Case no.	Initial method of cola administration	Methods of endoscopic treatment	Endoscopic procedure time (min)	No. of endoscopic sessions (*n*)	No. of used instruments (*n*)	Total amount of administered cola (liters)	Hospital stay (d)
CD group							
1	3 L drink			0	0	3	2
2	3 L lavage			0	0	3	2
3	3 L lavage			0	0	3	3
4	3 L lavage			0	0	3	2
PD group							
5	3 L drink	Mechanical lithotripsy	8	1	1	6	3
6	3 L lavage	Mechanical lithotripsy	45	1	1	6	3
7	3 L drink	Mechanical lithotripsy	14	1	2	6	2
8	3 L lavage	Mechanical lithotripsy	44	1	1	6	3
9	3 L drink	Mechanical lithotripsy	62	1	2	6	4
10	3 L lavage	Mechanical lithotripsy	42	1	1	6	3
11	3 L lavage	Mechanical lithotripsy	50	1	1	6	7
12	3 L lavage	Mechanical lithotripsy	30	1	1	6	3
13	3 L lavage	Polypectomy snare	52	2	2	9	3
14	3 L drink	Polypectomy snare	40	2	3	6	5
15	3 L drink	Polypectomy snare	58	2	3	9	5
16	3 L drink	Polypectomy snare	62	1	4	6	5
17	3 L drink	Polypectomy snare	72	2	5	6	3

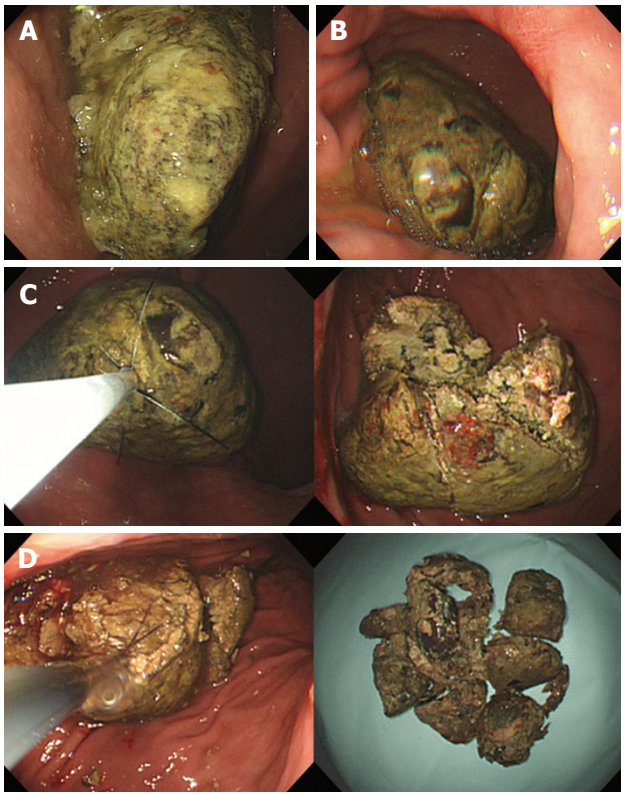

Figure 1 Endoscopic views of diospyrobezoar. A: Initial endoscopic view. A huge dark brownish-colored diospyrobezoar was noted in the stomach (case 8). B: Endoscopic view of one day after 3 L of cola lavage. The size of bezoar was decreased. C: Endoscopic procedure.The remnant bezoar was captured and fragmented into four pieces by basket. D: Endoscopic procedure. The fragmented bezoar was crushed and retrieved by grasping force.

dissolution was observed in four patients (23.5%), whereas 13 cases were partially dissolved with cola, i.e. their size was grossly decreased and their consistency was more softened than that observed before treatment (Figure 1A and B). The clinical characteristics of patients

Table 3 Comparison of clinical characteristics between complete dissolution and partial dissolution groups

	CD group (*n* = 4)	PD group (*n* = 13)	*P* value
Age, range (yr)	51-69	48-78	0.412
Gender (M:F)	0:4	5:8	0.208
Symptom duration, range (d)	14-21	7-90	0.624
Median (d)	20	21	
Type of bezoar			0.006
Phytobezoar	4	2	
Diospyrobezoar	0	11	
Size of bezoar			0.559
Over than 50% of stomach	2	5	
Less than 50% of stomach	2	8	
Endoscopic findings			0.241
GU	3	5	
Gastric outlet stenosis	1	8	
Method of administration of cola			0.441
Lavage	3	6	
Oral drink	1	7	

with complete dissolution (CD) and partial dissolution (PD) are compared and summarized in Table 2. The complete dissolution was observed in 4 out of 6 cases of phytobezoars, but no complete dissolution was noted in diospyrobezoars ($P = 0.006$). Other parameters, including age, gender, size of bezoar, endoscopic findings and the method of cola administration, were not different between the two groups (Table 3). Among the 13 cases with residual bezoars, eleven were completely treated with one endoscopic session and additional drink of three liters of cola. Four patients (case 13, 14, 15, 17) needed two endoscopic sessions to completely disrupt the bezoar.

Thirteen out of 14 (91.6%) patients were completely cured with a combination of cola and endoscopic treatment. The mean endoscopic procedure time was 44.53 ± 18.56 min and the mean number of used accessories was 2.07 ± 1.02. No procedure-

Table 4 Summary of cases for gastric phytobezoar treated with cola

Author	Type of bezoars	No. of cases	Methods of administration of Coca-cola	Duration of Coca-cola
Ladas *et al*[6]	Phytobezoar	5	3 L of Coca-cola lavage	12 h
Kato *et al*[7]	Diospyrobezoars	1	3 L of Coca-cola lavage	12 h
Chung *et al*[8]	Diospyrobezoars	1	Injection of 30 mL of Coca-cola and drinking 4 L of Coca-cola	2 d
Lin *et al*[9]	Diospyrobezoars	1	Injection and irrigation with 1 can of Coca-cola + oral drink of 1 can	
Okamoto *et al*[10]	Diospyrobezoars	1	Drinking 500 mL of Coca-cola per day	7 d
Sechopoulos *et al*[11]	Stump of vegetables	1	2 cans of Coca-cola injection	
Whitson *et al*[12]	Phytobezoar	1	Drinking 5 L of Coca-cola per day	5 d

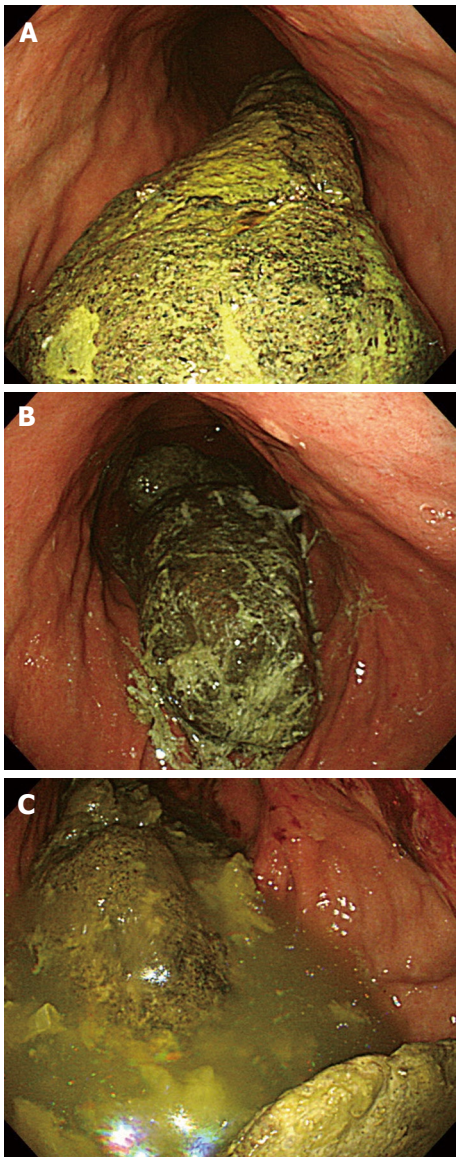

Figure 2 Endoscopic views of huge diospyrobezoar. A: Initial endoscopic view. Endoscopic view of huge dark brownish colored diospyrobezoar in the stomach (Case 17). B: Endoscopic view of one day after 3 L of cola lavage. The size was decreased and some fragmentation was observed. C: Endoscopic view of 4th day. The residual bezoar still remained with fragmentation (total amount of administrated cola was 9 L).

related complications developed, such as hemorrhage, perforation and small bowel obstruction. The mean hospital stay of all patients was 3.52 ± 1.32 d.

In another patient (case 17), the decreased size and softened consistency of the bezoar was observed after an initial lavage with three liters of cola lavage, but the endoscopic breakage failed (Figure 2A and B). So, 100 mL of cola were directly injected into the bezoar using an endoscopic needle and six additional liters of cola were administered orally for two days. On the fourth day, the bezoar still remained in place, with only partial resolution (Figure 2C). Because the patient refused an additional endoscopic treatment with cola, the bezoar was removed by surgery.

DISCUSSION

The treatment modalities for gastric bezoars include endoscopic therapy with fragmentation, medical treatment by enzymatic dissolution and surgery[13]. Various endoscopic methods and instruments for breaking up bezoars have been reported, including lithotripsy with basket[14], endoscopic suction removal with large-channel endoscopy[15], polypectomy snare[16] and biopsy forceps. However, these procedures are time-consuming. Furthermore, procedure-related complications may develop, such as bleeding, overtube-associated complications and intestinal obstruction caused by the fragmented, residual bezoars. In addition, chemical dissolution usually requires a long period of time and complications may develop, such as electrolyte imbalance, gastric ulcer and bleeding. The reported efficacy of chemical dissolution is variable[3,17].

Recently, Ladas *et al*[6] reported a five cases of gastric phytobezoars successfully dissolved by lavage with three liters of cola. Since then, there were six reports written in English and describing the treatment of gastric bezoars with cola. However, reports on cola dissolution have been limited to individual case reports. Methods and results are summarized in Table 4.

In this series, we report the clinical results of 17 gastric phytobezoars treated with cola. To our knowledge, this is largest study ever on this topic. The therapeutic efficacy of a lavage with three liters of cola (or of drinking the same amount), to achieve the complete dissolution of bezoars, was only 23.5%. Compared with previous reports, our success rate is very low. The reason for this low therapeutic efficacy of cola may be due to the fact that most cases of our series were diospyrobezoars (13 out of 17 cases, 76.4%), and in fact we failed to observe the complete dissolution of diospyrobezoars using cola alone.

Diospyrobezoars following ingestion of persimmon are formed by the agglutination of the tannins in the

skin of the fruit. Because of their hard consistency, endoscopic therapy with fragmentation or enzymatic dissolution is challenging and sometimes mechanical fragmentation cannot be accomplished. In a previous report, the efficacy of the combination of endoscopic fragmentation and pharmacotherapy was 80%[18]. There are four reports of cases in whom the complete dissolution of diospyrobezoars was carried out with cola (Table 4). The direct injection of small amounts of cola directly into the phytobezoars is also rapidly effective and safe[6,7,9]. However, in our experience, this technique was not effective for complete dissolving huge disopyrobezoar (case 17). So, in case of diospyrobezoars, complete dissolution might not be achieved with cola use alone.

Another reason for the low therapeutic efficacy may be the relatively short duration of cola administration. There were two successful dissolution cases with daily cola drinking for longer durations (7 d[10] and 3 mo[18]). A prolonged administration may have changed our clinical results, but it may also have induced metabolic disturbances due to the cola's high caloric content. Also, a longer administration time may prolong the *nil per os* time and the length of hospital stay. So, we used the short duration of cola and combined it with the endoscopic fragmentation.

Cola alone could not dissolve completely all gastric phytobezoars. However, in our series, softened-consistency or decreased size was observed in all residual bezoars. So, endoscopic fragmentation and retrieval of the bezoars could be easily performed. Except for four cases, all the procedures were completed in only one session, with relative short procedure time. As the bezoars' consistency was softened, disruption of accessories was prevented. So, these techniques are cost-effective when considering the length of the hospital stay, the number of endoscopic sessions and the used accessories. Also, using additional cola after endoscopic disruption may be helpful preventing small bowel obstruction due to daughter fragments.

Cola's mechanism of dissolution of bezoars is not well understood. The suggested mechanisms are: (1) the mucolytic effect of $NaHCO_3$, (2) the digestion of the bezoar by CO_2 bubbles and (3) the cola's acidity, which is similar to that of gastric acid[6].

In conclusion, the complete dissolution rate using three liters of cola was 23.5%, but no case of diospyrobezoars was completely dissolved. However, pretreatment with cola may be helpful and facilitate endoscopic fragmentation of gastric phytobezoars. A combination therapy of gastric phytobezoars with cola and endoscopic fragmentation is cost-effective and decreases the number of endoscopic sessions and accessories that are used as well as the hospital stay.

COMMENTS

Background

Ladas *et al* have first reported a case series of gastric phytobezoars that were successfully dissolved with cola lavage along with oral maintenance. Since then, there were several reports of gastric bezoar cases successfully treated only with cola.

Innovations and breakthroughs

The efficacy of cola for dissolving bezoars was very low compared with previous reports. There was no case of completely dissolved diospyrobezoar with cola. However, the use of cola for dissolving bezoars was cost-effective.

Applications

This study may suggest that dissolving gastric bezoars with cola alone is not the best treatment modality, especially for diospyrobezoars.

Peer review

The study evaluated the efficacy of cola treatment for gastric phytobezoars. The study is well written and perhaps the largest experience todate.

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BRIEF ARTICLES

TSPAN1 protein expression: A significant prognostic indicator for patients with colorectal adenocarcinoma

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Abstract

AIM: To determine if TSPAN1 overexpression is associated with clinicopathological and prognostic factors in human colorectal adenocarcinoma.

METHODS: Total RNA was extracted in 20 human adenocarcinoma tissues for TSPAN1 mRNA assay by RT-PCR. Eighty-eight specimens of human colorectal adenocarcinoma were surgically removed. TSPAN1 protein levels in cancer tissues were determined by immunohistochemistry using a polyclonal antibody against self-prepared TSPAN1. The correlation between TSPAN1 expression and the clinicopathological factors and the overall survival rate was analyzed by univariate and multivariate assay.

RESULTS: TSPAN1 mRNA was detected in 90.0% (18/20) of cancerous tissues. The light density of TSPAN1 mRNA expression levels was 0.89 ± 0.30 in adenocarcinoma by gel-image system. TSPAN1 protein expression was detected in 78.41% (69/88) and weakly expressed in 40% normal colorectal tissues. There were significant differences between colorectal adenocarcinoma and normal control epithelium ($P < 0.05$). TSPAN1 protein expression in colorectal cancerous tissue was significantly correlated with the histological grade, cell expression PCNA, lymph nodal metastasis and TNM staging of the disease. Patients with TSPAN1 protein overexpression had a significantly shorter survival period than that in patients with TSPAN1 protein negative or weak expression, respectively ($P < 0.05$). Furthermore, by multivariate analysis, TSPAN1 protein expression demonstrated an independent prognostic factor for human colorectal cancers ($P < 0.05$, relative risk 0.755; 95% confidence interval 0.302-1.208).

CONCLUSION: The expression of *TSPAN1* gene is increased in colorectal carcinoma, suggesting that TSPAN1 might serve as an independent prognostic factor for the colorectal adenocarcinoma patients.

Key words: TSPAN1; Colorectal adenocarcinoma; Semi-quantitative RT-PCR immunohistochemistry; Prognosis

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Chen L, Zhu YY, Zhang XJ, Wang GL, Li XY, He S, Zhang JB, Zhu JW. TSPAN1 protein expression: A significant prognostic indicator for patients with colorectal adenocarcinoma. *World J Gastroenterol* 2009; 15(18): 2270-2276 Available from: URL: http://www.wjgnet.com/1007-9327/15/2270.asp DOI: http://dx.doi.org/10.3748/wjg.15.2270

INTRODUCTION

The colorectal carcinoma is one of the most common malignant neoplasms, ranking the fourth frequency in men and third in women[1]. Although the prognosis has slightly improved in the past years, colorectal cancer is still the second and third major common cause of

cancer related death in men and women in the United States, respectively[2]. The incidence of colorectal cancer is the fourth in malignant tumor ranking in China, and it is increased dramatically in developing regions[3,4]. The colorectal cancer is thought to result from a combination of environmental factors, diet, lifestyle, chronic inflammation and accumulation of specific genetic alterations. The pathogenesis and development of colorectal cancer involve multi-genes and multi-steps. Ogino *et al*[5] showed the occurrence of colorectal cancer involved in a series of gene mutations, microsatellite instability (MSI) and 18q loss of heterozygosity (LOH). The other molecules studied include MST1 (Mammalian sterile 20-like kinase)[6]. Replication protein (RPA)[7], ELAV-like protein Huk and COX-2[8], α-catenin, β-catenin[9] α-ligatin, β-ligatin, Rho-a[10], *etc*. In fact, an established cascade of events leading to colorectal cancer development and progression is described by Vogelstein. The alteration of expression of these molecules often showed an obvious correlation with pathologic grading and clinical staging in colorectal cancer, which can be used as a biomarker for assessing prognosis. Currently, the assessment of prognosis is mainly based on pathological features of the tumor which is valuable to the triage of patients who will benefit from adjuvant therapy. The clinical pathological staging is the most popular standard prognostic approach for predicting the clinical outcome of colorectal cancer patients[11,12]. The prognosis of colorectal cancer is closely related to the tumor TNM stages. However, patients with similar stages of the disease have various outcomes. Therefore, there is a need to identify useful prognostic molecular markers in guiding treatment decisions and/or in developing more effective treatments. TSPAN1 (GenBank Accession No. AF065388) is a new member of TM4SF[13], which is located at chromosome 1 p34.1. It encodes a 241 amino acid protein. TSPAN1 was reported as a tumor-related gene recently[13-17]. In several studies, TSPAN1 gene over-expression was detected in liver cancer[14], prostate cancer[15], gastric carcinoma[16] and cervix cancer[17]. It has been proposed that TSPAN1 plays a role in cell mitosis and/or cause cell abnormal differentiation. In this study, we examined fresh tumor tissues and histological sections of colorectal adenocarcinoma to determine the expression of TSPAN1 mRNA and protein, and analyzed the relationship between the gene expression and clinicopathological parameters. The result suggests that overexpression of TSPAN1 is correlated to the prognosis of colorectal cancer patients.

MATERIALS AND METHODS

Specimen

A total of 88 patients with colorectal adenocarcinoma, diagnosed and treated from January 1998 to April 2000 were investigated in this study. Of the 88 cases evaluated, 46.6% (41 cases) were rectum cancers, 30.1% (27 cases) were sigmoid colon cancers, 6.8% (6 cases) were descending colon cancers, 2.3% (2 cases) were transverse colon cancers and 13.6 % (12 cases) were ascending colon cancers. The median age at the time of diagnosis was 62.2 years (range, 37-85). There were 50 male patients, 38 female patients. None of them had received chemotherapy or radiotherapy before diagnosis. After surgery, these patients with TMN stage Ⅱ took oral 5-fluorouracil and patients with stage Ⅲ-Ⅳ were subjected to 5-fluorouracil-based systemic chemotherapy. In order to avoid bias, each case was diagnosed by two pathologists.

The clinicopathological data were determined according to the WHO classification and TNM cancer staging[11,12,18]. The average size of the tumor was 4 cm (range from 1.5 to 7.6 cm), 54.5% (48 cases) were cauliflower/polyp type and 45.45% (40 cases) were ulcer/sclerotic type. Adenocarcinomas were graded predominantly on the basis of the extent of glandular appearances, and divided into well (lesions exhibit glandular structures in > 95% of the tumor, grade 1, 15.9% or 14 cases), moderate (lesions have 50%-95% glands, grade 2, 44.31% or 39 cases) and poor differentiation (lesions have 5%-50% glands, grade 3, 39.77% or 35 cases). Tumor limited in submucosa (T1) and muscularis propria (T2) as stage Ⅰ accounted for 32.95% (29 cases), tumor invaded through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues (T3) and tumor directly invades other organs or structures and/or perforates visceral peritoneum (T4) as stage Ⅱ accounted for 29.54% (26 cases), and the tumor with metastasis in 1-3 regional lymph nodes (N1-3) in any T as stage Ⅲ and the tumor with distant metastasis (M) in any T and N as stage Ⅳ, Ⅲ and Ⅳ accounted for 37.5 % (33 cases). Vascular invasion in 26 cases (29.55%) demonstrated that vessel wall was occlusive or infiltrating damaged up to the complete destruction with a surrounding fibroinflammatory reaction[19-21]. Such clinicopathological factors as perineural invasion and desmoplasia reaction were observed and analyzed as well. The proliferation level of cancer cells was evaluated based on the expression of PCNA in tumor parenchymas.

Semiquantitative reverse transcription-polymerase chain (RT-PCR)

Twenty cases of fresh colorectal cancer specimens were stored in -70℃ refrigerator immediately after dissection for semi-quantitative RT-PCR with co-amplification of TSPAN1 gene and an internal control β-actin. Briefly, total RNA from tumor tissues was extracted with TRLZOL reagent and the reverse transcription was performed with Rneasy Kit (Clontech, CA, USA) according to previously published protocols[14]. A 50 μL PCR reaction contains approximately 50 ng of human colorectal cancer ds-cDNA; 40 mmol/L Tricine-KOH, pH9.2; 15 mmol/L KOAc; 3.5 mmol/L Mg $(OAc)_2$; 0.2 μmol/L 5' TSPAN1 primer (5'-CAG-TTC-CCT-CTT-TCA-GAA-CTC-ACT-G-3'); 0.2 μmol/L 3' TSPAN1primer (5'-ATC-CAC-CCA-GAG-GCT-CTG-CTG-ATT-TCA-CCT-3'); 0.1 μmol/L 5' β-actin primer (5'-TTA-CAC-CCT-TTC-TTG-ACA-AAA-CCT-A-3');

0.1 μmol/L 3' β-actin primer (5'-CAA-AAG-CCT-TCA-TAC-ATC-TCA-AGT-3'); 0.2 mmol/L each of dATP, dGTP, dCTP and dTTP; and 1 μL of Advantage™ cDNA Polymerase Mix (50X; contains KlenTaq-1 and Deep Vent polymerases). The PCR cycling was as follows: PCR tubes were preheated at 94℃ for 20 s; then run 30 cycles at 96℃ for 6 s (denature); 60℃ for 20 s for annealing and 72℃ for 1 min for extension, in a DNA thermal cycle 9600 (PE Biosystems, CA, USA). PCR products were applied to electrophoresis on 1% agarose gel analysis; the expected *TSPAN1* gene was a band at 1159 bp. TSPAN1 expression was evaluated by calculating the average ratios of light density using symmetry computerized gel imaging system[14].

Immunohistochemistry

All 88 adenocarcinoma samples were routinely fixed in 40 g/L formaldehyde solution and embedded in paraffin. After slicing into 4 μm thick sections, immunohistochemistry was performed using Dako Elivision TM Plus Two-step System (PV-6000 kit, Zymed, Co., USA.). To detect the TSPAN1 and PCNA expressions in colorectal adenocarcinoma tissues, the sections were dewaxed in xylene and rinsed in alcohol and graded alcohol/water mixtures. Sections were then submitted to antigen retrieval treatment in a pressure cooker. The tissues were boiled in 0.01 mol/L, pH 6.0 citric acid buffer to retrieval antigen for 5 min. They were then treated with 0.3% hydrogen peroxide in absolute methanol to inhibit endogenous peroxidase activity for 15 min at room temperature. After blocking of background staining with diluted normal calf serum, sections were incubated overnight at 4℃ with polyclonal antibodies against TSPAN1 (antibody prepared with the help of American San Francisco gene biotechnology company) and PCNA (PC10, No. 40780708, DAKO, USA), respectively. Subsequent reaction proceeded using a two step assay, immunoreaction was visualized with peroxidase-3,3'-diaminobenzidine (DAB). Finally, sections were lightly counterstained with Mayer's haematoxylin and mounted. The negative controls were set by omitting the primary antibodies. The positive controls were the hepatocellular carcinoma with positive expressions of TSPAN1. In addition, 10 specimens from the marginal normal mucosa of tumor were used as normal controls[16].

Evaluation of immunohistochemical staining

All sections were blindly analyzed by two experienced pathologists under light microscope. Based on the estimated percentages of positive parenchyma cells and/or the immunostaining intensity, which was determined by comparing the immunoreactivity of the positive controls that were included in each experiment, staining results were divided into four categories: (-) tissues specimens: positive parenchyma cell with less than 5% of the cancer tissues and/or weakly stained; (+) tissue specimens: positive parenchyma cell with less than 25% of the cancer tissues and/or weakly stained; (++) tissues specimens: positive parenchyma cell with less than 50% of the cancer tissues and/or moderately stained, and (+++) tissue specimens: positive parenchyma cell with more than 75% of the cancer tissues and/or strongly stained[14,16].

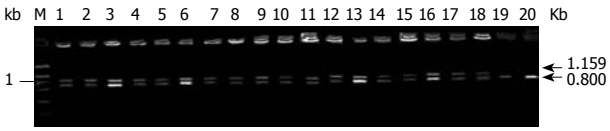

Figure 1 Analysis of TSPAN1 and β-actin mRNA expression in 20 cases of colorectal adenocarcinoma. TSPAN1 and β-actin mRNA expressions were detected in 20 cases of colorectal adenocarcinoma tissues by semi-quantitative RT-PCR. The upper bands were TSPAN1 and the lower bands were β-actin. Lane M: 200 ng of 1 kb size ladder (New England BioLabs); Lanes 1-20: Colorectal adenocarcinoma tissues.

Statistical analysis

Association between *TSPAN1* gene expression and other clinicopathological factors of the tumor were assessed by the Fisher's exact test (two-sided) for categorical variables and χ^2 test were used to compare ordinal variables. The grading-related data was analysed by Spearman test. Overall survival was defined as the period from the date of diagnosis to the date of death. Survival curves were determined according to the Kaplan-Meier method, and compared using Log-rank test statistical differences. Multivariate survival analysis was performed with SPSS version 11.0 Software (Chicago, IL, USA).

RESULTS

RT-PCR detection of TSPAN1 mRNA expression

Total RNA was extracted from 20 cases of colorectal adenocarcinoma tissues. RT-PCR analysis of TSPAN1 mRNA expression was then performed. The positive rate of TSPAN1 mRNA expression was 90% (18/20) in the colorectal adenocarcinoma (Figure 1), and the relative amount of TSPAN1 mRNA levels in cancer tissues was assessed based on the β-actin control. The relative amounts of TSPAN1 mRNA were 0.89 ± 0.30.

Immunochemistry detection of TSPAN1protein expression

TSPAN1 was mainly presented in cytoplasm and located at membrane as well. In the normal control epithelium, 3 cases presented a weakly positive staining of TSPAN1, and only 1 case presented moderately positive expression (Figure 2A). We observed TSPAN1 protein expression in 78.41% (69/88) cases of tumors, in which 17.39 % (12/69) was displayed as strong expressed (+++), 44.93% (31/69) as moderately expressed (++), and 37.68% (26/69) as weakly expressed (+). There were significant differences between colorectal adenocarcinoma and normal control epithelium ($P < 0.05$), (Figures 2B-E).

Correlation with clinicopathological parameters

To investigate the role of TSPAN1 expression in colorectal cancer, we examined the correlation of

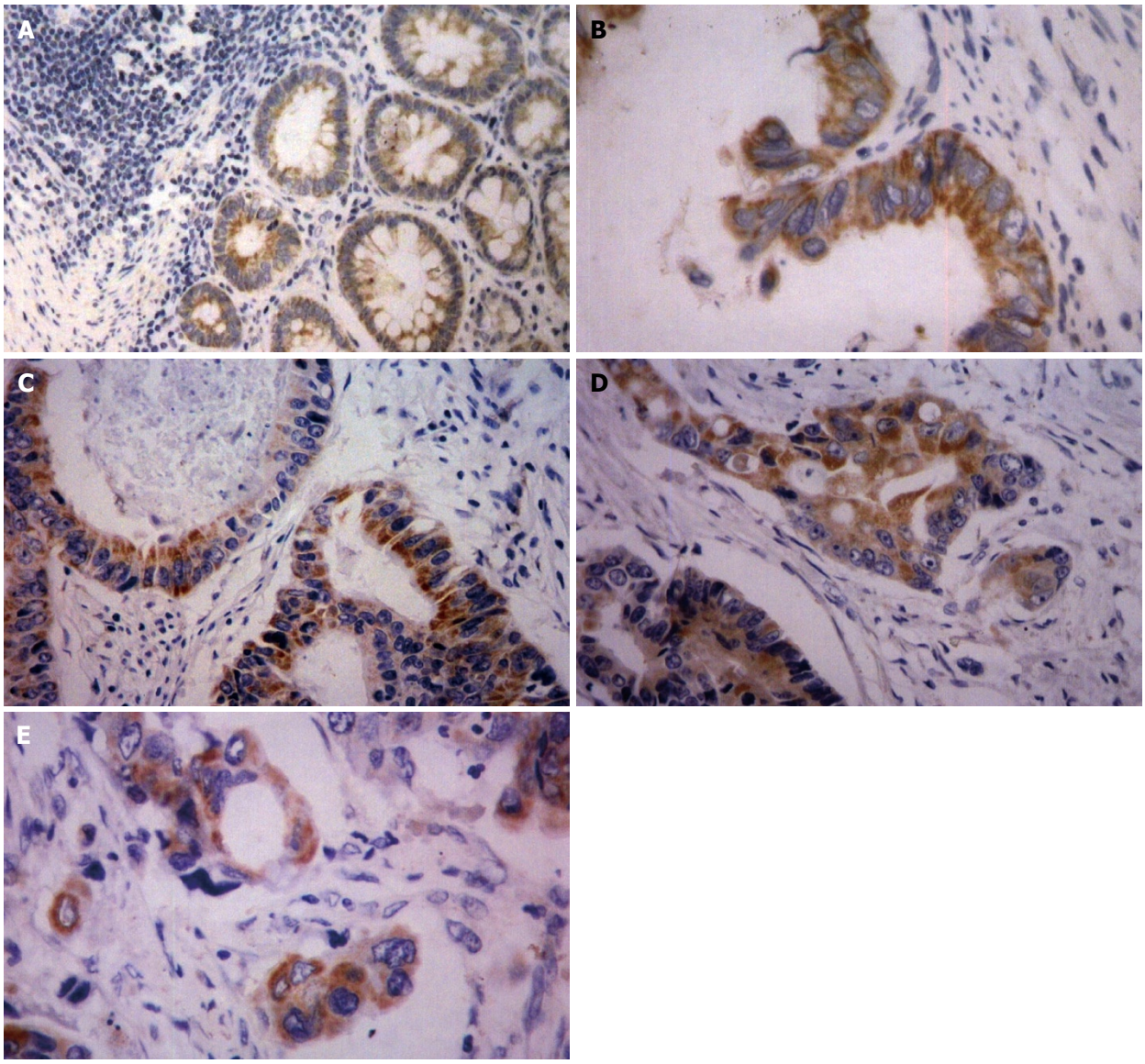

Figure 2 TSPAN1 expression in normal tissues (A), colon cancer tissues (B, C), rectal cancer tissues (D, E). Paraffin section of human colorectal carcinoma tissues was stained with anti-TSPAN1 polyclonal antibody by immunohistochemistry. A: TSPAN1 weakly expressed in the cytoplasm. (× 100). B, C: TSPAN1 was located in the cytoplasm with yellow granulation. (× 200). D, E: Cancer nest showed positive TSPAN1 expression and vascular invasion. (× 200).

TSPAN1 expression with the clinicopathological features (Table 1). We found a positive correlation with histological grade, PCNA expression, nodal metastasis and TNM stages (P = 0.001, 0.015, 0.008 and 0.002, respectively). TNM staging of colorectal cancer is more important for patient's prognosis evaluation. The five-year survival rate of TMN stage 1 is more than 95%, while it is less 10% in patients with TNM stage Ⅲ-Ⅳ. From Table 1, it can be found that the TSPAN1 expression rate and intensity in early TNM stage were lower than in late TNM stage cancer tissues. In addition, TSPAN1 expression was not associated with vascular invasion, perineural invasion and desmoplasia.

Correlation with patients' survival rate

Within a period of 60 mo of the follow-up, 24 cancer-related deaths occurred, 3 of the deaths come from 9 patients with TSPAN1 negative tumors, and 21 from 33 patients in the TSPAN1 positive group. In the entire cohort, the overall survival rate of patients with TSPAN1 negative tumors were significantly higher than that of those with TSPAN1 positive tumors (63.64% *vs* 33.33%; log-rank test: χ^2 = 15.48, P = 0.001). Kaplan-Meier estimated the overall survival rate based on cell TSPAN1 expression in the patients with a follow-up period of 60 mo (Figure 3). To compare with other clinicopathological factors, the effects of histologic grades, node status, PCNA expression, TNM stages, vascular invasion or perineural invasion on the patients' survival were also analysed with univariate log-rank test. As shown in Table 2, the factors of cellular differentiation, node status, PCNA expression, TNM stages had a significant effect on the overall survival rate (P = 0.03, 0.001, 0.0003 and 0.002, respectively). Furthermore, univariate survival analysis was performed to investigate possible prognostic impact of TSPAN1 in

Table 1 Correlation of clinicopathological parameters with TSPAN1 expression

Parameters	Cases	TSPAN1 expression intensity				*P*
		-	+	++	+++	
Gender						
Male	50	9	14	21	6	0.472
Female	38	10	12	10	6	
Tumor size (cm)						
< 4.0	35	10	10	10	5	0.469
> 4.0	53	9	16	21	7	
Type						
Cauliflower/polyp	48	9	14	16	9	0.595
Ulcer/infiltration	40	10	12	15	3	
Location						
Rectum	41	9	11	17	4	0.595
Colon	47	10	15	14	8	
Grade						
Well	14	6	6	2	0	0.001
Moderate	39	9	14	14	2	
Poor	35	4	6	15	10	
PCNA						
+	43	14	14	13	2	0.015
++/+++	45	5	12	18	10	
Lymph node metastasis						
No	55	16	20	14	5	0.008
Yes	33	3	6	17	7	
TNM stage						
Ⅰ	29	11	9	7	2	0.002
Ⅱ	26	5	11	7	3	
Ⅲ-Ⅳ	33	3	6	17	7	
Vascular invasion						
No	62	14	21	20	7	0.424
Yes	26	5	5	11	5	
Perineural invasion						
No	67	16	22	22	7	0.235
Yes	21	3	4	9	5	
Desmoplasia						
No	55	10	16	20	9	0.647
Yes	33	9	10	11	3	

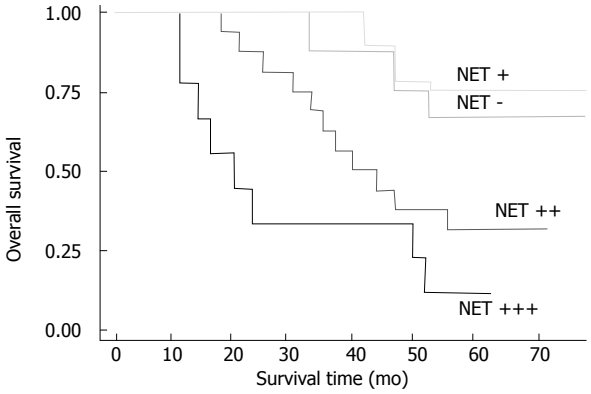

Figure 3 Overall 5-year survival curve of colorectal adenocarcinoma patients with TSPAN1 negative (-) and TSPAN1 positive (+, ++, +++) for the entire cohort (*P* = 0.001) was estimated by Kaplan-Meier test. Survival rate in TSPAN1 expression groups (++, +++) were obviously lower than that of weak expression (+) or negative (-) group, respectively ($P < 0.05$). There was no significant difference of survival rates between TSPAN1 negative group (-) and TSPAN1 weak expression group (+).

colorectal cancer. As shown in Table 2, the expression of TSPAN1 correlated with a worsening of the survival probability, which was statistically significant. This was also confirmed by a multivariate survival analysis including above factors (Table 3). All of these results suggested that TSPAN1 expression in tumors was an independent prognostic factor for colorectal adenocarcinoma patients (relative risk = 0.755; 95% confidence interval: 0.302-1.208 $P = 0.001$).

Table 2 Univariate analysis by Log-rank test

Parameters	5-yr survival rate (%)	Log-rank test	
		χ^2	*P*
TSPAN1 expression			
-	66.67 (6/9)	15.48	0.0015
+	71.4 (5/7)		
++	35.3 (6/17)		
+++	11.1 (1/9)		
Grade			
Well	87.5 (7/8)	6.91	0.0316
Moderate	37.5 (6/16)		
Poor	27.8 (5/18)		
Node status			
No	71.6 (12/17)	15.67	0.0001
Yes	24.0 (6/25)		
PCNA expression			
+	63.1 (12/19)	9.05	0.0026
++-+++	26.1 (6/23)		
TNM stages			
Ⅰ	83.3 (10/12)	16.20	0.0030
Ⅱ	62.5 (5/8)		
Ⅲ-Ⅳ	13.6 (3/22)		
Vascular invasion			
No	46.4 (13/28)	1.39	0.2377
Yes	37.7 (5/14)		
Perineural invasion			
No	44.7 (14/32)	0.77	0.3795
Yes	40.0 (4/10)		
Desmoplasia			
No	33.3 (3/9)	0.02	0.8829
Yes	42.4 (15/33)		

Table 3 Multivariate analysis in Cox proportional hazard model

Variable	Multivariate analysis				*P* value
	HR	SD	Z	95% CI	
TSPAN1 expression	0.755	0.231	3.27	0.302-1.208	0.001
Grade	0.798	0.318	2.51	0.175-1.421	0.012
Node status	1.779	0.509	3.49	0.781-2.778	0.000
PCNA expression	1.325	0.475	2.79	0.394-2.256	0.005
TNM stages	1.159	0.341	3.39	0.490-1.829	0.001
Vescular invasion	0.491	0.423	1.16	0.338-1.320	0.246
Perineural invasion	0.409	0.473	0.87	0.517-1.336	0.386
Desmoplasia	0.061	0.415	0.15	0.752-0.873	0.884

DISCUSSION

Many studies reported that TSPAN1 mRNA and protein were expressed in human normal tissues and carcinomas[13-17]. Serru detected TSPAN1 expression in various cell lines by RT-PCR including cervical cancer, lung cancer, squamous carcinoma, colorectal cancer and breast cancer cells[13]. Wollscheid *et al*[17] detected TSPAN1 mRNA level by RT-PCR and TSPAN1 protein by immunohistochemistry in cervical cancer and found that the gene was expressed in CIN Ⅲ, cervical squamous cell carcinoma and adenocarcinoma,

especially in all undifferentiated cervical carcinoma and adenocarcinoma. They thought *TSPAN1* gene expression correlated to cell proliferation and may be used as a marker for cervical cancer prognosis. However, *TSPAN1* gene expression in human colorectal cancer tissues has not been reported so far. In this study, we for the first time demonstrated that TSPAN1 mRNA and protein were extensively expressed in 90% and 78% human colorectal cancer tissues, respectively. Our results revealed that epithelial cells of the normal colon or rectum displayed a slight expression of TSPAN1 antigen (Figure 2A). There was significant difference between cancer tissues and normal control. The results are consistent with most other reported data[12-14] and suggest that the TSPAN1 expression is a specific marker for malignant transformation.

In colorectal cancer, the presence of many tumor-associated antigens and their relationship with clinical pathological parameters have been described[22-23]. PCNA, a major marker for cell proliferation, is highly expressed in most tumors[24]. In this study, the finding of a significant positive correlation between TSPAN1 and PCNA expression provided further evidence to support a potential role of TSPAN1 in tumor proliferation process (Table 1). The colorectal cancer development may hence relate to the accumulation of TSPAN1 protein in tumor cells. Similarly, our previous study found that TSPAN1 expression correlated with tumor proliferation maker Ki67 expression in human gastric carcinomas[16].

Currently, the TNM stage represents the main tool for identifying prognostic differences among patients with colorectal cancer. The reported 5-year survival rate is 95% for stage Ⅰ patients, 67% for stages Ⅱ, and 9.4% for stage Ⅲ and Ⅳ patients[25]. In our prospective 5-year follow-up study, the overall survival rate was 83.3% for stage Ⅰ patients, 62.5% for stage Ⅱ patients, and 13.6% for stage Ⅲ and stage Ⅳ patients (Table 2). Similarly, we showed that there was a significant correlation between the overall survival rate and the disease stages. Our study revealed that there was a statistically significant association between TSPAN1 expression and the various stages of colorectal cancer, in which TSPAN1 positive staining was seen in 63.64% patients with shorter survival time (Table 3). The univariate and multivariate analyses suggested that TSPAN1 status, PCNA expression, tumor stages and nodal status were strong predictors for the final clinical outcome (Table 3). Likewise, another study in our lab also showed that TSPAN1 expression was significantly correlated with the metastasis and poor prognosis of gastric carcinoma[16]. Increasing TSPAN1 protein expression was found associated with more advanced stages of cervical carcinoma[17]. All these findings suggest that TSPAN1 over-expression status might yield unfavorable prognosis for some types of cancers. Identifying those patients with high-risk colorectal cancers by TSPAN1 expression detection would be of great benefit for improving the treatment strategies. By the way, other reports displayed that vascular invasion and perineural invasion were correlated with a poor prognosis[19-21], but in this study we found no direct effect on tumor prognosis.

Colorectal carcinoma is one of the most common cancers in western world and in China, however its molecular mechanism is still unclear. To understand the specific regulation of gene expressions between colorectal cancer and non-cancer tissues and know the genes or proteins characteristics will delineate the molecular changes and obtain useful diagnostic marker. We have demonstrated that TSPAN1 was expressed in majority of human colorectal carcinomas in the current study. TSPAN1 expression, measured by immunohistochemistry in the tumor tissues, may be a candidate gene for diagnosis and prognosis of colorectal carcinoma. The overexpression of TSPAN1 in cytoplasm is associated with higher tumor grade, metastasis, proliferation, and more advanced stages and poor prognosis in colorectal adenocarcinoma patients, suggesting a tumor-related gene role of TSPAN1 in human colorectal cancer development.

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COMMENTS

Background

The colorectal cancer results from a combination of environmental factors, diet, lifestyle, chronic inflammation and accumulation of specific genetic alterations. TSPAN1 (GenBank Accession No. AF065388) is a new member of TM4SF located at chromosome 1 p34.1. It encodes a 241 amino acid protein. TSPAN1 was reported as a tumor-related gene recently.

Research frontiers

TSPAN1 gene over-expression was detected in liver cancer, prostate cancer, gastric carcinoma and cervix cancer. It has been proposed that TSPAN1 plays a role in cell mitosis and/or cause cell abnormal differentiation.

Innovations and breakthroughs

In this study the authors examined fresh tumor tissues and histological sections of colorectal adenocarcinoma to determine the expression of TSPAN1 mRNA and protein, and analyzed the relationship between the gene expression and clinicopathological parameters, and found that overexpression of TSPAN1 is correlated to prognosis of colorectal cancer patients.

Applications

Testing TSPAN1 expression in tissues would be a useful tool to evaluate the prognosis of patients with colorectal cancer.

Peer review

The authors examined the expression of Net-1 in colorectal tissues, a novel gene whose function has yet to be understood so far. This study is of some clinical significance.

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CASE REPORT

Jejunoileal bypass: A surgery of the past and a review of its complications

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Author contributions: Singh D conceived the idea; Singh D wrote the paper; Laya AS developed the tables and edited the figures; Allen MJ and Clarkston WK analyzed the paper and revised it critically for important intellectual content; Clarkston WK provided the funding.

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Abstract

Jejunoileal bypass (JIB), popular in the 1960s and 1970s, had remarkable success in achieving weight loss by creating a surgical short bowel syndrome. Our patient had an unusual case of liver disease and provided no history of prior bariatric surgery. Later, it was recognized that he had a JIB in the 1970s, which was also responsible for the gamut of his illnesses. Patients with JIB are often not recognized, as they died of complications, or underwent reversal of their surgery or a liver-kidney transplant. Early identification with prompt reversal, and the recognition and treatment of the life-threatening consequences play a critical role in the management of such patients.

Key words: Jejunoileal bypass; Bariatric surgery; Weight loss; Obesity; Morbid obesity

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INTRODUCTION

Bariatric surgery is one of the few proven methods that cause durable weight loss. Failure of conservative means of producing permanent weight reduction in patients with morbid obesity, led to the introduction of operative approaches, such as jejunoileal bypass (JIB), which became popular in the late 1960s and early 1970s[1].

At that time, JIB was the most effective surgical intervention for achieving and maintaining weight loss. Typically, 35 centimeters of proximal jejunum was anastamosed, end-to-side or end-to-end, to the terminal 10 centimeters of ileum[2] (Figure 1). It was presumed that patients undergoing this procedure would experience continued hyperphagia, but would accomplish weight loss due to malabsorption[3]. As a result of JIB, patients whose preoperative weight was over 157 kg lost a mean of 58 kg at the end of 1 year[2].

However, JIB surgery has long been abandoned as a method of weight reduction surgery because of serious short and long-term complications. The number of patients who currently retain a jejunoileal bypass is small, as most patients have died or undergone reversal of their operation or conversion to a different bariatric procedure[3]. Recognition of previous JIB and understanding of its metabolic consequences are essential in the proper management of these patients.

CASE REPORT

A 64-year-old male was admitted on a regular basis for tense ascites (requiring serial large volume paracentesis) attributed to underlying advanced liver disease of unclear etiology. It was presumed to be the result of steatohepatitis from nonalcoholic fatty liver disease and/or chronic hepatic congestion due to decreased cardiac function.

The patient had a prior history of morbid obesity (191 kg, BMI 62) and cholecystectomy. His physical examination was remarkable for jaundice, abdominal ascites, spider angioma in the upper chest, gynecomastia and splenomegaly.

He had numerous other medical problems including multiple kidney stones with three previous lithotripsy interventions, progressive kidney disease requiring hemodialysis, a 30-year history of intermittent loose

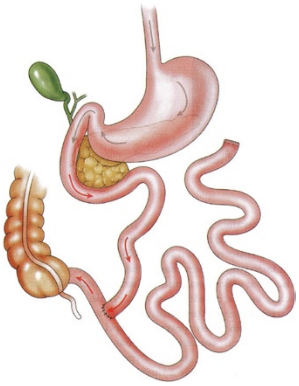

Figure 1 Jejunoileal bypass. A surgical short bowel syndrome created by bypassing more than 90% of the functioning small intestine.

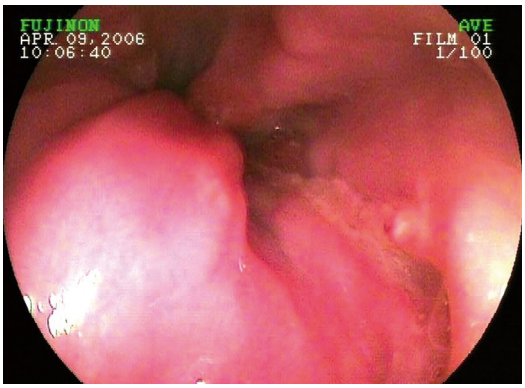

Figure 3 Upper endoscopy showing grade I esophageal varices, as a result of portal hypertension.

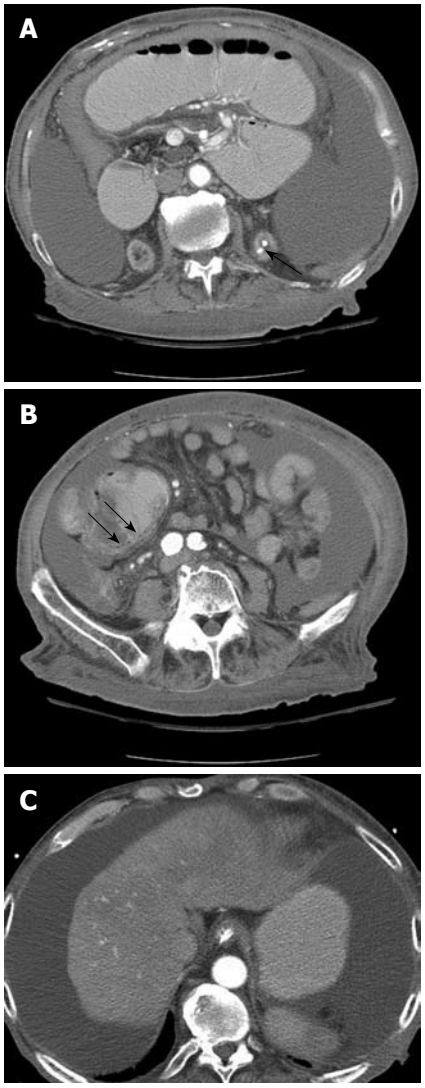

Figure 2 CT scan of the abdomen. showing stones in the left kidney (A, arrow), pneumatosis intestinalis (B, arrow) and shrunken nodular liver with abdominal ascites (C).

Table 1 Laboratory investigations

BUN/Creatinine (normal = 8-26/0.9-1.3)	34/8.2
Albumin (normal = 3.5-5.0)	1.9
Prothrombin time/INR (normal = 11.9-14.3/< 1.0)	25.3/2.4
ALT/AST (normal = 15-41/14-63)	45/41
Platelet count (normal = 140-400)	129
Serum albumin ascites gradient	2.3
Hepatitis profile (A, B, C)	Negative

BUN: Blood urea nitrogen; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

stools, arthritis, fatigue, paresthesias, progressive loss of night vision, joint pains, slurred speech, incoordination and weakness.

On further questioning, the patient and family revealed that he had "weight loss surgery" in the 1970s at another facility. However, an upper endoscopy showed an intact pylorus, which raised suspicion of possible previous JIB surgery. His archived medical records were then obtained and it revealed that the patient indeed had a jejunoileal bypass performed in 1974.

Initial laboratory evaluation is noted in Table 1. Further evaluation revealed deficiency of all fat-soluble vitamins. The markers of other causes of chronic liver disease (viral hepatitis B and C, antinuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, ceruloplasmin, iron and ferritin) were negative. Stool studies showed findings consistent with steatorrhea. Computed tomography imaging of his abdomen revealed nephrolithiasis, pneumatosis intestinalis, a shrunken and nodular liver with abdominal ascites, and osteopenia (Figure 2). Upper endoscopy, as in Figure 3, revealed esophageal varices, portal hypertensive gastropathy and no evidence of prior gastric bypass.

He underwent a transjugular liver biopsy that showed portal fibrosis (stage 2, grade 1) with specimen fragmentation. A repeat transjugular liver biopsy with hepatic hemodynamic measurements was performed, with a hepatic portal venous gradient at 7 mmHg (normal, 1-4 mmHg), the wedge pressure was 23 (normal, 4-13 mmHg) and the hepatic vein pressure was 16 mmHg (normal, 2-10 mmHg) - findings consistent with portal hypertension.

The patient declined reversal of JIB and did not wish evaluation for liver-kidney transplantation. He is currently being managed symptomatically by hospice care.

The complications of JIB are summarized in Table 2 above[4].

Table 2 Complications of jejunoileal bypass

Problem	Mechanism	Manifestations in this patient
Steatohepatitis Possible cirrhosis	Amino acid deficiency	Advanced liver disease with portal hypertension
Renal oxalosis	Excess oxalate absorption; Oxalate not bound by calcium	Multiple kidney stones and three previous lithotripsy interventions; Progressive kidney disease due to suspected oxalate nephropathy requiring lifelong hemodialysis
Fat soluble vitamin deficiency	Malabsorption; Steatorrhea	Serum levels: Vitamin A = 17 (360-200 mg/L) Vitamin D ⩽ 10 (22-67 pg/mL) Vitamin E = 3 (5.5-17.0 mg/L) Vitamin K ⩽ 0.03 (0.1-.2 ng/mL)
Gallstones	Bile acid loss; Mobilization of cholesterol	Previous cholecystectomy for symptomatic cholelithiasis
Enteritis	Bacterial overgrowth	30 years of diarrhea and steatorrhea Pneumatosis intestinalis
Arthritis	Bacterial toxin; Autoimmune	Bilateral knee and shoulder pain
Fatigue syndrome	Vitamin deficiency; Multifactorial	Marked fatigue, bed-ridden status
Bypass encephalopathy	Possible deficiency; Possible D-lactic acid deficiency	Slurred speech, incoordination and weakness
Bypass dermatitis	Possible antigen-antibody complex (enteric bacteria)	Cutaneous urticarial rash

CONCLUSION

Although jejunoileal bypass was effective and reliable, it was associated with severe complications such as renal failure (37%), diarrhea (29%) and consequent electrolyte imbalances, calcium oxalate nephrolithiasis (29%), liver disease (10%), fat-soluble vitamin deficiencies, malnutrition and death[5]. The most severe early complication of JIB was acute liver failure (7%)[5]. Our patient exhibited nearly all of the known metabolic complications described in the literature (Table 2).

Of the various types of bariatric procedures, JIB is particularly devastating because of its dramatic complications, and is no longer used for the management of morbid obesity. A number of studies[6-8] highlighted the high complication rates even after a decade and sometimes lifelong, difficulty maintaining satisfactory follow-up and the need for frequent revision surgery making it an unacceptable procedure performed remotely on any major scale.

Today, this procedure is a formidable diagnostic challenge because it was a surgery of the olden days (1960s and 1970s), old records may not be available and upper endoscopy may be essentially normal. Most patients have either died or had conversion to a different bariatric procedure. The importance lies in early identification based on clinical history, with prompt reversal and the recognition and treatment of the life-threatening metabolic consequences.

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CASE REPORT

Embolization of an unusual metastatic site of hepatocellular carcinoma in the humerus

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. This case documents an unusual metastatic presentation of HCC in the humerus. Preoperative palliative arterial embolization of the tumor was performed to arrest severe tumor bleeding caused by the biopsy. Embolization turned out to be useful also in limiting/preventing potential uncontrolled bleeding during subsequent amputation.

Key words: Hepatocellular carcinoma; Humerus; Upper arm; Metastasis; Embolization

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Hansch A, Neumann R, Pfeil A, Marintchev I, Pfleiderer S, Gajda M, Kaiser WA. Embolization of an unusual metastatic site of hepatocellular carcinoma in the humerus. *World J Gastroenterol* 2009; 15(18): 2280-2282 Available from: URL: http://www.wjgnet.com/1007-9327/15/2280.asp DOI: http://dx.doi.org/10.3748/wjg.15.2280

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world[1]. The most frequent sites of extrahepatic metastases of HCC are the lungs, lymph nodes, bones, and adrenal glands[2], whereas the extremities, and especially the humerus, are very rare metastatic sites. Here we report an interesting case of humerus metastasis of HCC with severe hemorrhage after biopsy.

CASE REPORT

A 74-year-old man with a background of alcoholism and smoking initially presented with pain on the right side of the chest. Clinical examination revealed mild hepatomegaly and pallor. An abdominal ultrasound showed the presence of a solid mass in the right lobe of the liver, measuring 5.2 cm × 6.5 cm, with heterogeneous echotexture (Figure 1A). Computed tomography (CT) imaging of the abdomen confirmed the presence of a lesion in segment Ⅷ of the liver, with central areas of necrosis (Figure 1B). An additional, smaller focal lesion was found also in segment Ⅶ of the liver (Figure 1C). Both lesions showed typical imaging characteristics of an HCC. Notably, a thrombosis of the portal vein was also present (Figure 1D), which presented a contraindication for chemoembolization of the HCC. The patient was discharged.

After 4 mo, the patient was referred again with progressive painful swelling of the left upper arm and superficial ulceration. An X-ray showed a destructive lesion of the left humerus, associated with a bulging soft tissue component (dimensions 5.5 cm × 4 cm). The diaphysis of the humerus was completely destroyed for a length of 4 cm (Figure 2A and B). Magnetic resonance imaging (MRI) clearly demonstrated a large osteolytic lesion (coronal dimensions 6.2 cm × 5 cm) in the left humerus space, with complete destruction of the bone (Figure 2C and D). The tumor reached the surface of the skin. A surgical biopsy was performed to sample tissue from the ulcerated mass. However, massive bleeding developed from the biopsy site immediately after excision. Because the hemorrhage could not be stopped by electrocauterization, it was decided to perform an arterial embolization on the assumption that possible HCC metastases would typically be hypervascularized. A 5 French cobra catheter was advanced from the femoral artery into the left axillary artery and a selective

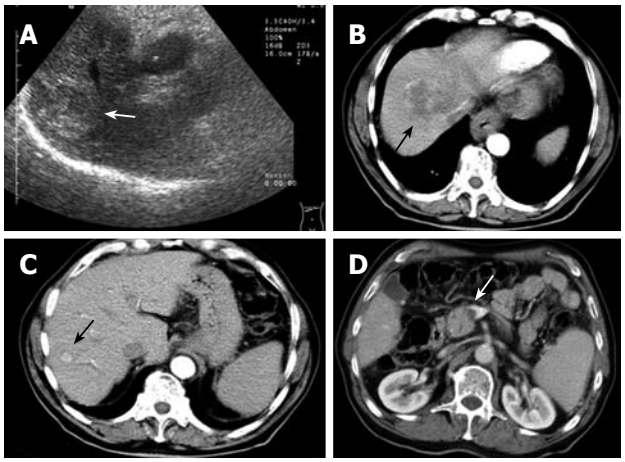

Figure 1 Liver sonography (A) and CT imaging of hepatocellular carcinoma (HCC) (B-D). A: Round but inhomogeneous tumor with hyper- and hypo-echoic appearance in the sonography (diameter 6 cm, arrow); B: Axial multidetector CT contrast image at the arterial phase demonstrated a tumor lesion in the right upper lobe (arrow). Dimension 7 cm × 6.5 cm × 60 cm with hyperdense periphery and hypodense centre (latter areas of necrosis); C: Additional 1.4 cm × 1 cm × 1 cm hyperintense lesion in segment Ⅶ of the liver (arrow); D: Partial thrombosis of the portal vein (arrow).

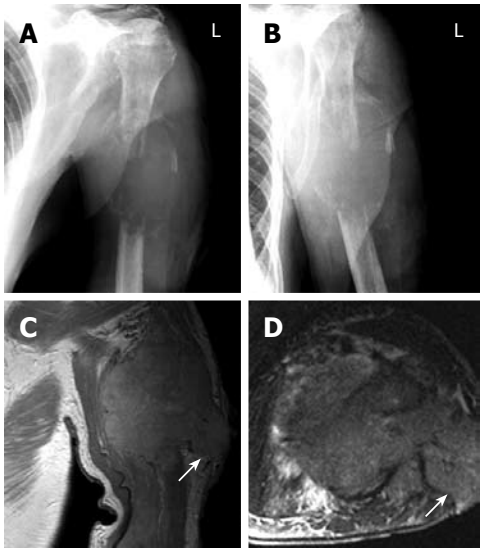

Figure 2 X-Ray (A, B) and MR imaging (C, D) of HCC metastasis at the humerus. A and B: Destructive lesion in the left humerus associated with a bulging soft tissue component, 5.5 cm × 4 cm in dimension; C: Coronal noncontrast PD-weighted (TR/TE, 1920/12) image demonstrating a soft tissue tumor (coronal dimension 6.2 cm × 5 cm) with complete destruction of the humerus, the tumor reached the surface with ulceration (arrow); D: Axial noncontrast TIRM sequences (TR/TE, 7400/92) show the edematous tumor in the centre of the extremity and the lateral tumor branch to the surface (arrow). In this region, a biopsy was taken which was followed by massive hemorrhage.

arteriogram was obtained. Injection of contrast medium showed a hypervascular, destructive tumor of the humerus (Figure 3A). Extravasation of the contrast medium indicated the presence of hemorrhage (Figure 3B). Afterwards, multiple accessible feeding branches were reached with superselective catheterization using a tracker catheter, and embolized with Bead Block (size 300-500 μm, Terumo Europe, Leuven, Belgium). Follow-up angiographies during embolization showed stasis of flow within the tumor. At

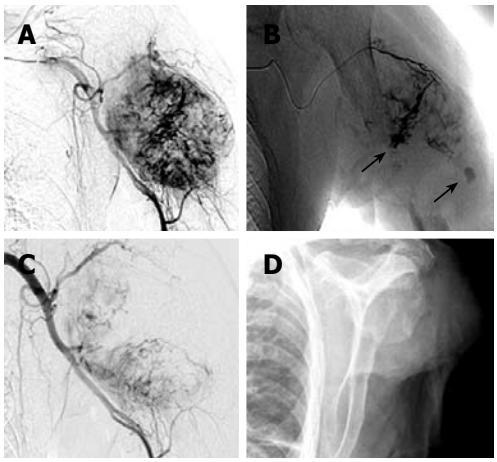

Figure 3 Catheter angiography of the tumor before (A, B) and after embolization (C), X-Ray after final treatment with amputation of the left upper extremity (D). A: Catheter angiography of the axillary artery reveal a round hypervascular tumor; B: Selective angiography of a tumor feeding vessel as side of application of Bead Block (size 300-500 μm, Terumo Europe, Leuven, Belgium) for embolization; C: After embolization of main parts of the tumor, at this status no areas of bleeding detectable but small parts of the tumor still perfused; D: Final treatment with amputation of the left upper extremity.

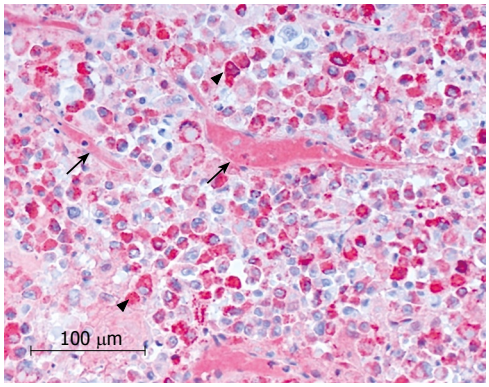

Figure 4 Neoplastic cells of HCC metastasis were diffusely stained by hepatocellular antigen (for instances, see arrowheads), destroyed bone areas within the tumor are visible (arrows).

the end, partial tumor embolization was achieved. The medial parts of the tumor remained perfused, since not all the small-sized branches of this region could be accessed or localized (Figure 3C). However, bleeding was successfully stopped. There were no postprocedural complications. On the following day, amputation of the left arm was deemed necessary since an alternative treatment, e.g. osteosynthetic stabilization of the destroyed humerus, was not achievable (Figure 3D). Thus, *a posteriori* the embolization proved useful also as a measure to prevent uncontrolled hemorrhages during amputation.

The histopathological evaluation of the biopsy confirmed the diagnosis of metastasis from the liver HCC (Figure 4).

DISCUSSION

This report describes an unusual case of massive metastasis of a HCC carcinoma to the upper arm, with partial

destruction of the humerus. To the best of our knowledge, no such cases have been reported in the literature to date. Also, while bone metastases of HCC usually manifest as multiple lesions, no other bone metastases were detected in this case.

The prognosis of HCC patients with extrahepatic metastasis is generally poor[3]. It is estimated that 30%-78% of HCC show metastases at autopsy[4]. HCC spreads mainly *via* the hematogenous route, causing intra- and extra-hepatic metastases that are generally hypervascular and, if located in the bone, osteolytic. Hypervascularity should be taken into account before biopsy excisions since the procedure can cause an uncontrolled hemorrhage, as reported here and by Chen *et al*[5] who described a life-threatening hemorrhage from a sternal metastasis of HCC. In the present case, the marked size of the tumor in the upper arm did not allow us to distinguish whether the metastasis had primarily localized in the muscle or in the bone. However, intramuscular HCC metastases are extremely rare[6,7], possibly because of the contractile action of muscle, its local pH environment, and/or the accumulation of lactic acid. Also, skeletal muscle produces tumor suppressors which may contribute to the rarity of metastases in the muscles[8]. A study with 194 autopsies of malignant tumors showed that macro- or micro-metastases of skeletal muscle were present only in 34 cases (17.5%)[9]. Metastases of HCC are clearly more frequent in the bone but, unlike the present humerus case, their most common sites are the vertebrae, the pelvis, and the ribs. In turn, bone metastases of HCC are commonly characterized by expansive soft tissue masses with bone destruction, as in the present case.

To date, only a few reports are available concerning arterial embolization of bleeding HCC metastases. This procedure can support treatment to reduce the tumor size[10] or to reduce the severity of the symptoms (e.g. pain or bleeding). Wallace *et al*[11] reported that patients with bone metastases who underwent embolization had a reduction of pain for 4-9 mo. In our case, the goal of embolization was to control the bleeding following biopsy excision. A posteriori, a benefit was also the containment/prevention of potential bleeding during amputation. Amputation became necessary because of the advanced tumor size and the degree of bone destruction.

In conclusion, this case documents an unusual metastatic presentation of HCC in the humerus. Preoperative palliative arterial embolization of the tumor was performed to arrest severe tumor bleeding caused by the biopsy. Embolization turned out to be useful also in limiting/preventing potential uncontrolled bleeding during subsequent amputation.

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CASE REPORT

Repair of a mal-repaired biliary injury: A case report

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Abstract

Iatrogenic bile-duct injury post-laparoscopic cholecystectomy remains a major serious complication with unpredictable long-term results. We present a patient who underwent laparoscopic cholecystectomy for gallstones, in which the biliary injury was recognized intraoperatively. The surgical procedure was converted to an open one. The first surgeon repaired the injury over a T-tube without recognizing the anatomy and type of the biliary lesion, which led to an unusual biliary mal-repair. Immediately postoperatively, the abdominal drain brought a large amount of bile. A T-tube cholangiogram was performed. Despite the contrast medium leaking through the abdominal drain, the mal-repair was unrecognized. The patient was referred to our hospital for biliary leak. Ultrasound and cholangiography was repeated, which showed an unanatomical repair (right to left hepatic duct anastomosis over the T-tube), with evidence of contrast medium coming out through the abdominal drain. Eventually the patient was subjected to a definitive surgical treatment. The biliary continuity was re-established by a Roux-en-Y hepatico-jejunostomy, over transanastomotic external biliary stents. The patient is now doing well 4 years after the second surgical procedure. In reviewing the literature, we found a similar type of injury but we did not find a similar surgical mal-repair. We propose an algorithm for the treatment of early and late biliary injuries.

INTRODUCTION

Laparoscopic cholecystectomy (LC) has emerged as a gold standard of cholecystectomy and is the commonest laparoscopic surgical procedure performed by many surgeons worldwide. Unclear anatomy of the biliary tract and acute cholecystitis are associated with an elevated risk of bile duct injuries. These are serious surgical complications and are sometimes unrecognized during the procedure. A clear interpretation of the biliary anatomy as well as a good surgical experience are prerequisites for a definitive surgical repair. Primary surgical repair and further misinterpretation of the biliary anatomy with consequent mal-repair of the biliary tract injury are very unusual conditions. We present here a case of biliary tract injury that was recognized during LC but that was submitted to a mal-repair surgical procedure causing difficult problems of correct interpretation and management for the definitive surgical repair.

CASE REPORT

A 45-year-old woman was admitted to the referring hospital with symptomatic gallbladder stones for elective LC. She had not undergone any previous operations. Cholecystectomy was reported to be difficult by the operating surgeon. He reported that she had acute cholecystitis, and the biliary anatomy was not clear. During the operation he recognized that he caused a biliary injury, and for this reason he decided to convert

the intervention to open surgery. He completed the cholecystectomy, and performed a repair of the biliary injury over a T-tube, according to his interpretation of the injury. He did not perform a cholangiogram before the repair, but the post-repair intraoperative cholangiogram was interpreted as a good repair. An infra-hepatic drain was inserted. During the first postoperative day, the abdominal drain brought out 500 mL of bile and the T-tube drained 100 mL of a similar fluid. On the following days, the abdominal drain brought 800-1000 mL of bile daily and the T-tube brought 40-60 mL daily. During the next 2 wk, the output through the drains did not decrease, and the treating surgeon asked to transfer the patient to our hospital, with a diagnosis of biliary fistula. The referring surgeon had an average experience in laparoscopic surgery. Past medical history revealed a non-insulin-dependent diabetes mellitus on oral medication.

In our hospital, the patient appeared pale, mildly dehydrated, not jaundiced and with normal vital signs. She was in the average weight range. The abdominal examination revealed a right sub-costal scar, two scars from the trocars, and two drains (an abdominal drain and the other was a T-tube) that both contained bile. She did not look septic.

The laboratory results showed: hemoglobin 10 g/dL; white blood cell count 11 300/mm^3; K^+ 3.2 meq/L; Na^+ 132 meq/L. Liver enzymes and bilirubin were within the normal range.

Abdominal US showed normal liver size and echogenicity, without intra-hepatic biliary dilatation, a small sub-hepatic collection, and sub-hepatic multiple clips. The distal part of the biliary tree was not identified.

A T-tube cholangiogram (Figure 1) was done in our hospital, which showed a grade Ⅳ Bismuth injury, with the T-tube limbs within the left and right hepatic ducts. The biliary contrast medium appeared immediately through the abdominal drain, and the distal part of the biliary tree was not shown. These findings (mal-placed T-tube and fistula) suggested a plan for the definitive biliary surgical repair. We did not perform magnetic resonance cholangiography (MRC), because the patient already had a T-tube, and also she had a sub-hepatic collection.

Informed consent was obtained from the patient and her family. She was given a prophylactic third-generation cephalosporin, which was continued for 24 h postoperatively. She was shifted to insulin therapy from the day she was admitted to our hospital, which was continued until she returned to her normal diet, on postoperative day 7. The intraoperative finding was a Bismuth grade Ⅳ injury that was repaired by right and left hepatic duct end-to-end anastomosis over a T-tube, performed by the referring surgeon. During exploration, we found an absence of the biliary confluence and the common hepatic duct, which may have indicated a misinterpretation by the first surgeon of the common hepatic duct as the cystic duct, therefore, he excised this region altogether with the gallbladder. Multiple clips on the distal biliary tree (common bile duct) were found.

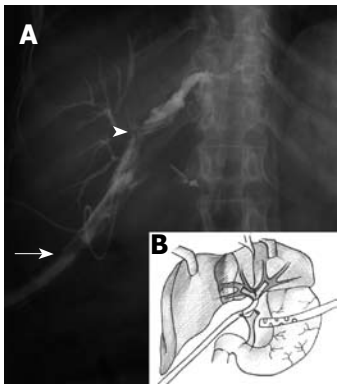

Figure 1 T-tube cholangiography. A: Postero-anterior T-tube cholangiogram showing biliary tract mal-repair (arrowhead indicates the T-tube, arrow indicates the tube drain); B: Schematic diagram of the cholangiogram.

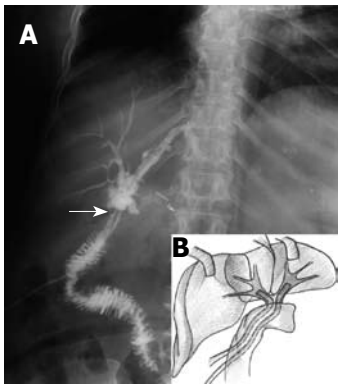

Figure 2 T-tube cholangiography. A: Postero-anterior view of the T-tube cholangiogram, which was done in our hospital, after the definitive surgical repair of the biliary tract injury (arrow: two intra-bilio-jejunal stents); B: Schematic diagram of the cholangiogram.

We also noted a bile leak from the posterior wall of the anastomosis that was performed previously over the T-tube. The T-tube was removed and a Roux-en-Y hepatico-jejunostomy over two trans-anastomotic stents was then performed (Figure 2). The patient had a good postoperative recovery without complications. The trans-stents' cholangiogram performed on postoperative day 10 showed patent anastomosis without leakage. The stents were closed and the patient was discharged. The tubes were removed after 2 mo. The patient was followed up by clinical examination, liver enzymes and abdominal ultrasound (US), every 3 mo for the first year, every 6 mo for the second year, and then every year thereafter. In her final follow-up visit in August 2008, she was in good condition, 4 years after our surgical repair. She did not have any wound complications, neither in the early nor in the late postoperative period.

DISCUSSION

Misinterpretation of biliary anatomy was the main cause of biliary mal-repair. The hard task was to understand this mal-repair carried out by the referring surgeon, and then to perform a definitive biliary repair. The only possible explanation was that the referring surgeon misinterpreted the left hepatic duct as the common bile duct. This was very hard to recognize from the first radiological study, performed in the referring hospital, in which the contrast medium spilled out into the abdominal drain, and it was interpreted incorrectly as the common bile duct. As a result of the poor quality of this radiological study, we performed another T-tube cholangiogram and discussed the case with our

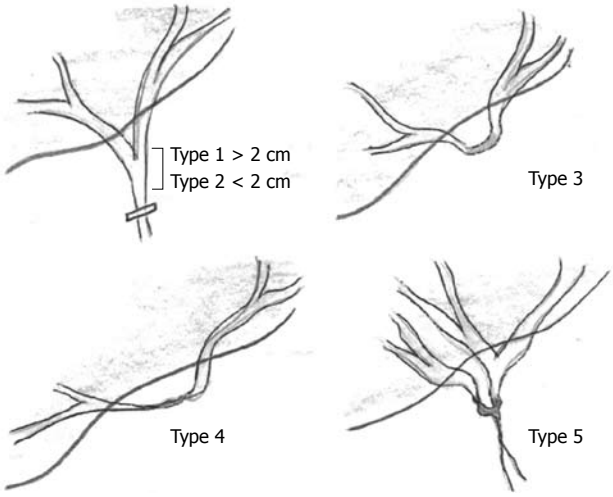

Figure 3 Bismuth classification of biliary injuries.

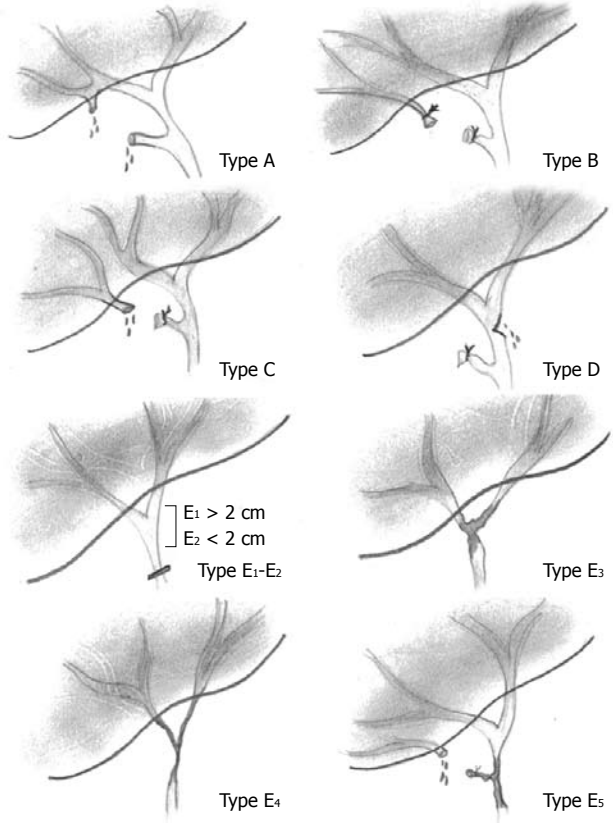

Figure 4 Strasberg classification of biliary injuries.

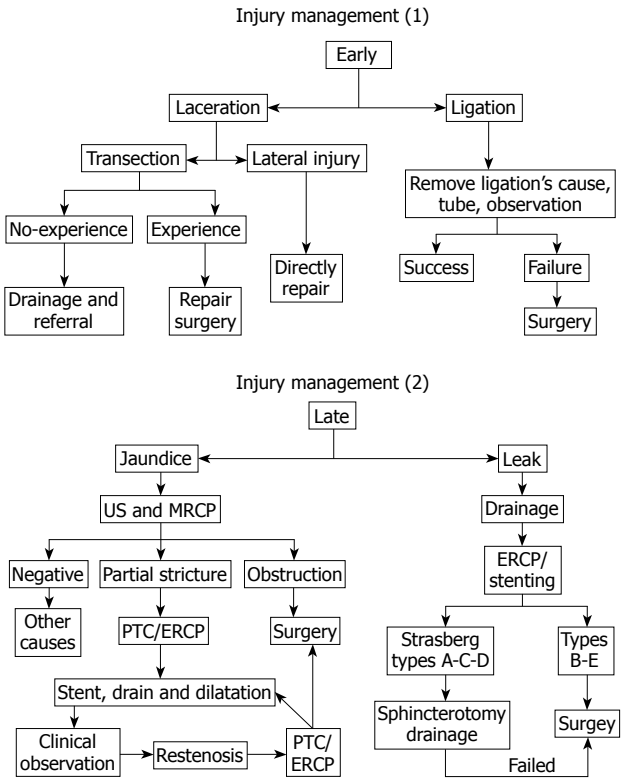

Figure 5 Our suggested flow chart for the management of early and late presentations of biliary injuries. PTC: Percutaneous transhepatic cholangiography; ERCP: Endoscopic retrograde cholangiopancreatography.

radiologist, who confirmed our suspicion of the mal-repair. Although this kind of injury is well known (Bismuth type Ⅳ), we could not find a similar case of mal-repair in the English literature.

The incidence of biliary tract injury ranges from 0.2% to 0.8% worldwide, and even an experienced surgeon can cause such injury[1-5]. There is more than one classification for biliary injury, but the most widely used for surgical purposes are the Bismuth and Strasberg classifications (the later includes the former).

Our patient presented with grade Ⅳ Bismuth or Strasberg E Ⅳ biliary injury, which required surgical treatment. Figures 3 and 4 show drafts for both Bismuth and Strasberg classifications[4,6,7].

The referring surgeon tried to repair the injury immediately during the first operation. The identification of injury during surgery is reported to be between 15% and 50% of cases. Although primary repair is possible, and can be done laparoscopically, it should only be performed by an experienced surgeon. If this is not the case, drainage and referral to an experienced surgeon is the rule[2,3,8-10]. Poor identification of the anatomical features of the hepatic triangle represents the commonest cause of injury[3]. Bismuth noted that the level of the stricture found during repair was one level higher than the level of injury identified during the first operation[7]. During the re-operation for failed repair, the level of stricture has been reported to be even higher[11].

In our case, we re-established the biliary continuity by a Roux-en-Y hepatico-jejunostomy, after trimming the previous biliary anastomosis, over two stents inserted into the right and left hepatic ducts. A postoperative tube cholangiogram is shown in Figure 4. Different methods of repair have been reported by different surgical teams[5,7,12]. Percutaneous and endoscopic retrograde cholangiopancreatography (ERCP) interventions can both be used in the management of biliary injuries, but under different conditions than ours[13]. We recommend, in cases that present with suspected stricture, to start with MRCP as the procedure of choice to identify the anatomy and the type of injury. In our specific case, the patient already had a drain in her biliary system, and she also had a biliary leak, therefore, it was appropriate to

use these drains for delineating the biliary anatomy by a tube cholangiogram.

We propose an algorithm (Figure 5) for the treatment of early and late biliary complications. We consider complications that appeared during the first month postoperatively as early (usually leaks), and those presenting after one month as late complications (usually strictures).

Bile duct injuries are serious surgical complications. A major study led by Cameron showed post-repair mortality of 1.7%, with a similar percentage of patients dying before repair, as a result of sepsis. The study of Cameron also showed a complication rate after repair of 42.9%, despite an early referral to a specialist center[12]. Long-term outcome after repair showed good results in > 90% of cases[8].

In conclusion, biliary tract injuries are sometimes difficult to recognize, even for experienced surgeons. In the absence of an experienced surgeon, it is mandatory to limit the surgical manipulation to simple drainage, and to refer the patient to a more specialized center, in order to give the best chance for definitive treatment.

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CASE REPORT

Mucosal Schwann cell "Hamartoma": A new entity?

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Abstract

Schwannoma is a well-described, benign nerve sheath tumor of the soft tissue, but is rare in the gastrointestinal tract. Gastrointestinal schwannomas are often incidentally discovered as small polypoid intraluminal lesions. In this report, we describe the clinicopathologic and immunohistochemical features of a distinctive neural mucosal polyp composed of a diffuse cellular proliferation of uniform bland spindled cells in the lamina propria that entraps the colonic crypts. Immunohistochemical analysis revealed strong and diffuse positivity for the S-100 protein. To avoid confusion of these solitary colorectal polyps containing pure spindled Schwann cell proliferation in the lamina propria with neural lesions that have significant association with inherited syndromes, it is better to use the designation "mucosal Schwann hamartoma".

Key words: Nerve sheath tumors; Gastrointestinal Schwannoma; Hamartoma

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INTRODUCTION

Schwannoma is a common soft tissue tumor, but it appears to be rare among spindle cell mesenchymal tumors of the gastrointestinal tract[1-4]. Colorectal schwannomas are uncommon, and are incidentally discovered as small polypoid intraluminal lesions, often with mucosal ulceration, during colonoscopy screening. Gastrointestinal schwannomas have characteristic histological features that are different from their soft tissue counterparts, such as the presence of a reactive lymphoid peripheral cuff, the absence of encapsulation and degenerative changes[5-7]. The tumors are mainly situated in the muscularis propria of the digestive wall. Rectal bleeding, colonic obstruction, and abdominal pain are the most common presenting symptoms. The separation of GI stromal tumors (GISTs) from gastrointestinal schwannoma is clinically important because the former group have a high risk of malignant behaviour[8-11], while the second are benign. Recently the designation "mucosal Schwann cell hamartoma" has been proposed for lesions containing diffuse pure Schwann cell proliferation in the lamina propria, which entrap adjacent crypts, to avoid confusion with the neural lesions that are associated with inherited syndromes such as von Recklinghausen's neurofibromatosis[12]. In this study, we report a case of a colorectal polyp comprising diffuse Schwann cell proliferation in the lamina propria that belongs to the entity proposed.

CASE REPORT

Clinical presentation

A 60-year-old woman with no personal history of malignancy underwent a colonoscopy during the workup of occult blood in the stool. She had no family history of colon cancer and no history of familiar adenomatous polyposis, multiple endocrine neoplasia type Ⅱb, neurofibromatosis type Ⅰ, or Cowden syndrome.

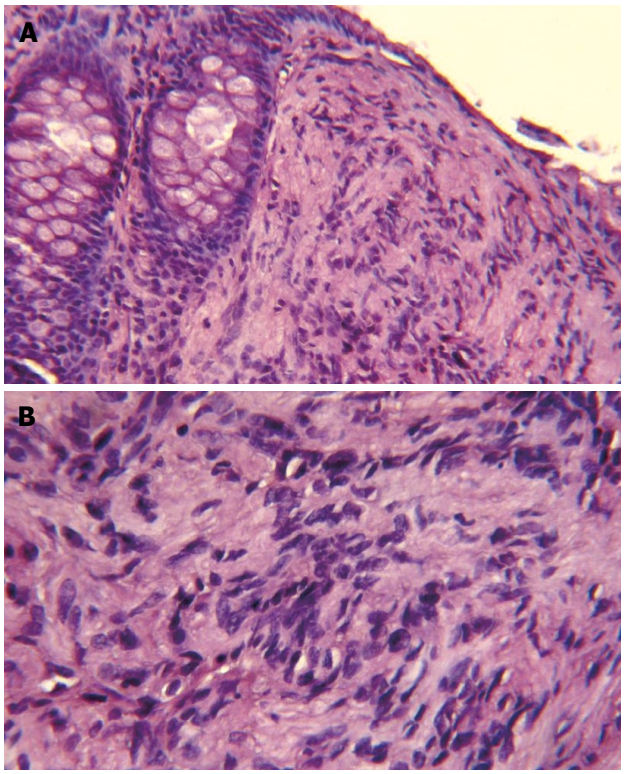

Figure 1 Histological features of the lesion. Low- (A) and high (B)-power magnification of haematoxylin and eosin stained tissue sections of colorectal mucosa. A diffuse, Schwann cell proliferation in the lamina propria, which entraps colonic crypts is visible. Cytologically, the lesions are composed of uniform bland spindle cells with elongated nuclei, dense eosinophilic cytoplasm, and minimal intervening stroma with vague Verocay bodies.

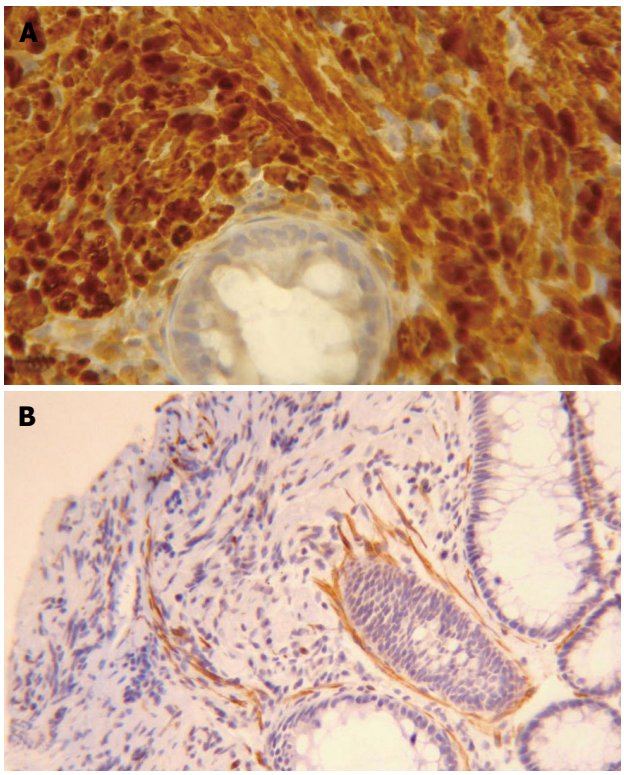

Figure 2 Immunohistochemical staining. The lesion consists of a pure population of Schwann cells, as shown by the diffuse immunoreactivity for the S-100 protein (A). Only scattered myoepithelial cells and vascular structures were highlighted by the immunostaining for α-smooth muscle actin (B).

Endoscopic and microscopic findings

Endoscopic examination showed a small sessile polyp of 0.5 cm in diameter without mucosal ulceration in the rectosigmoid colon. A biopsy was obtained. Hematoxylin and Eosin stained histologic sections showed a diffuse cellular proliferation of uniform spindle cells with elongated, tapering nuclei, and indistinct cell borders, arranged in whorls and vague Verocay bodies, entrapping adjacent crypts (Figure 1). The epicenter of the lesion was located in the lamina propria without involvement of the muscularis mucosae. No nuclear atypia, pleomorphia, or mitoses were seen. The immunohistochemical analysis demonstrated that all the cells were extensively and strongly positive for the S-100 protein (Figure 2A). Cells were negative for CD117 (KIT), α-smooth muscle actin (1A4) (Figure 2B), and CD34 (QBEND/10). Scattered mild chronic inflammation with the rare appearance of mast cells was present in the background.

DISCUSSION

Schwannoma (or neurilemoma) is an encapsulated nerve sheath tumour, common in the soft tissue. In the gastrointestinal tract schwannomas are rare and non-encapsulated, although well circumscribed[3]. They may appear as a small intramucosal nodular lesion, polypoid lesions, or poorly demarcated transmural proliferations[13]. According to the reports of Hou *et al*[7] and Daimaru *et al*[1] schwannomas were more frequently found in the stomach than in the colon or rectum. Miettinen and colleagues[3] and Lewin and colleagues[14], have described and characterized the largest series of colorectal schwannomas. Spindle cells variants have been found to be the most frequent, but epithelioid and plexiform schwannomas have also been described.

Herein we described a case of colorectal polyp in a female patient discovered during colonoscopy for the workup of occult blood in the stool. Histological examination of the polyp revealed a poorly circumscribed proliferation of uniform bland spindle cells, arranged in whorls and with a poorly formed area suggestive of Verocay bodies. The epicenter of the lesion was in the lamina propria, without involvement of the muscularis mucosae. Cellular spindle cell proliferation was interspersed between colonic crypts. No peripheral lymphoid aggregates, nuclear atypia nor mitosis were seen. The proliferated cells were strongly S-100 positive, and CD117, α-smooth muscle actin and CD34 negative, corresponding to the Schwann cell phenotype.

Mucosal neural polyps with similar features to our case have been recently described by Gibson *et al*[12], and we agree with author's proposal of the new and interim designation of "mucosal Schwann cell hamartoma" for this lesion, to avoid confusion with the gastrointestinal neural lesions that have significant associations with inherited syndromes[15-17]. Our case should not be considered as an intramucosal schwannoma, although qualitatively composed exclusively of Schwann cells, because

it presents peculiar histological features: the lack of circumscription, the absence of a peripheral lymphoid cuff[5], and crypt entrapment. Solitary ganglioneuromas and perineuriomas might predominantly involve the mucosa but the population of ganglion cells and the positivity for epithelial membrane antigen (EMA) are distinctive features. In contrast to neurofibromas, the polyp evaluated in our study was cytologically uniform, and, based on diffuse immunoreactivity for S-100 protein, seems to be composed essentially of a pure population of Schwann cells. Moreover, CD34 and neurofilament are useful stains for the differential diagnosis, because neurofibromas typically demonstrate a significant subpopulation of CD34-positive stromal cells and scattered axons.

Leiomyomas might also be encountered arising in association with the muscularis mucosae of the colon. They express desmin, calponin, and caldesmon but lack S-100 protein[14].

It is important in the GI tract to recognize GI stromal tumors (GISTs), which might pursue a malignant course. GISTs might have neural differentiation but they are typically reactive with c-kit/CD117 antibodies, and originate from or differentiate into the interstitial cells of Cajal with activating mutations in the KIT24-27 and PDGFRa genes[7-10].

In conclusion, the lesion we have encountered should be categorized as a mucosal Schwann cell hamartoma. Accurate histological differential diagnosis of this kind of lesion has clinical relevance, not only for immediate patient management, but also because it might provide the first clue to the existence of inherited tumor syndromes (searching for ganglion cells), which will have broader implications for the patient's family and potentially important consequences for genetic counselling.

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CASE REPORT

Fibrosing cholestatic hepatitis following cytotoxic chemotherapy for small-cell lung cancer

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Abstract

Fibrosing cholestatic hepatitis (FCH) is a variant of viral hepatitis reported in hepatitis B virus or hepatitis C virus infected liver, renal or bone transplantation recipients and in leukemia and lymphoma patients after conventional cytotoxic chemotherapy. FCH constitutes a well-described form of fulminant hepatitis having extensive fibrosis and severe cholestasis as its most characteristic pathological findings. Here, we report a case of a 49-year-old patient diagnosed with small-cell lung cancer who developed this condition following conventional chemotherapy-induced immunosuppression. This is the first reported case in the literature of FCH after conventional chemotherapy for a solid tumor. In addition to a detailed report of the case, a physiopathological examination of this potentially life-threatening condition and its treatment options are discussed.

Key words: Fibrosing cholestatic hepatitis; Immunosuppression; Chemotherapy; Lung cancer; Hepatitis B virus; Lamivudine

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INTRODUCTION

Hepatitis B virus (HBV) is a well-known pathogen which can cause fulminant hepatitis in some patients undergoing cytotoxic chemotherapy. Patients who have completely recovered from acute hepatitis can harbor a latent HBV infection for decades[1].

Fibrosing cholestatic hepatitis (FCH) is a recognized unique variant of viral hepatitis which was originally reported in 1991 by Davies *et al*[2] in HBV-infected recipients of liver allografts. Other authors have also reported FCH cases in HCV-infected recipients after liver transplantation[3] and in renal[4], cardiac[5] or bone marrow transplanted patients[6] as well as in patients with acquired immunodeficiency syndrome[7]. FCH has additionally been reported secondary to hepatitis C allograft reinfection[8].

However, to our knowledge only 3 cases of FCH have been reported after conventional cytotoxic chemotherapy. Nonetheless, all of them were diagnosed with non-solid tumors (acute myelogenous leukemia[9], acute lymphoblastic leukemia[10] and low grade non-Hodgkin's lymphoma[11]). FCH is associated with extremely high mortality[12].

We report a case of a patient diagnosed with small-cell lung cancer who developed FCH under chemotherapy-induced immunosuppression.

CASE REPORT

This is a case of a 49-year-old male with a 38 pack-year smoking history and a past medical history of hepatitis B. He had not received previous blood transfusions. At the time of admission, the patient presented with pain in the right hemithorax. Initial investigations included thoracic X-ray, thoracic computed tomography (CT) and positron emission tomography (PET)-CT imaging demonstrating right pleural effusion, right upper lobe

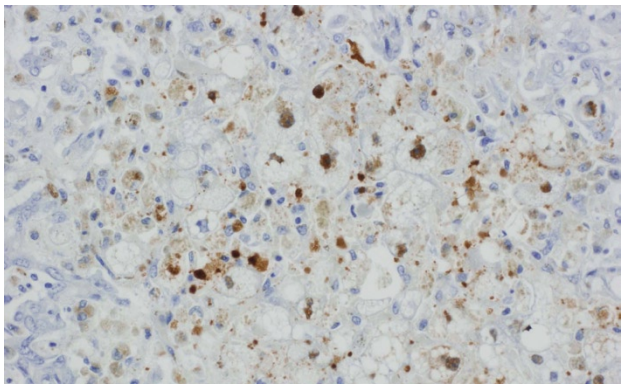

Figure 1 Liver necropsy stained with antibody against hepatitis B core antigen. FCH is also characterized by massive expression of hepatitis B core antigen (nuclear and cytoplasmic).

atelectasia, mediastinal adenopathies and liver and bone metastases. After a bronchoscopy-guided biopsy, he was diagnosed with extensive-disease small-cell lung cancer. The patient accepted chemotherapy and a combination regimen of cyclophosphamide 400 mg/m^2 (days 1-3), adriamycin 40 mg/m^2 (day 1), cisplatin 100 mg/m^2 (day 2), vincristine 2 mg (day 3) and etoposide 100 mg/m^2 (days 1-3) was administered. After the first cycle, the patient presented with grade Ⅳ febrile neutropenia in spite of pegfilgrastim prophylaxis. For this reason, a 20% dose reduction was applied on the second cycle. Doses were escalated to the original levels in the third cycle, during which the patient presented with elevation of glutamic oxalacetic transaminase (GOT) (232 UI/mL) and glutamic pyruvic transaminase (GPT) (639 UI/mL).

Following the third cycle, a PET-CT scan showed a radiological complete response. In contrast, the patient showed persistent elevation of transaminases (GOT: 198 UI/mL; GPT: 400 UI/mL).

After administration of the fourth cycle, he developed grade 4 thrombocytopenia and neutropenia. A complete serological study was performed demonstrating hepatitis B reactivation (HBsAg, HBeAb and HBcAb IgG positive). Despite treatment with the nucleoside analogue entecavir and methylprednisolone, the hepatitis progressed towards acute hepatic failure (GOT: 1879 UI/mL; GPT: 2663 UI/mL; total bilirubin: 42 mg/dL; direct bilirubin: 37 mg/dL; indirect bilirubin: 5 mg/dL; prothrombin time: 10%). A liver biopsy was not performed, due to thrombocytopenia and clotting time elevation.

The patient was transferred to the Intensive Care Unit and received plasma exchange, vasoactive drugs and wide spectrum antibiotics (piperacillin-tazobactam). In spite of these therapies, he died a month after the last chemotherapy cycle from acute liver failure.

The autopsy showed residual tumor in the mediastinum and peribronchial nodes, a hepatic reaction and fibrosing cholestatic hepatitis. The microscopic study revealed marked hepatocyte degeneration, whereas the immunohistochemical analysis demonstrated hepatocytes diffusely stained for HBsAg following both intracytoplasmic and cytoplasmic membranous patterns and nuclear and intracytoplasmic HBcAg staining patterns (Figure 1). Serum levels of HBV DNA polymerase were 17 857 100 UI/mL.

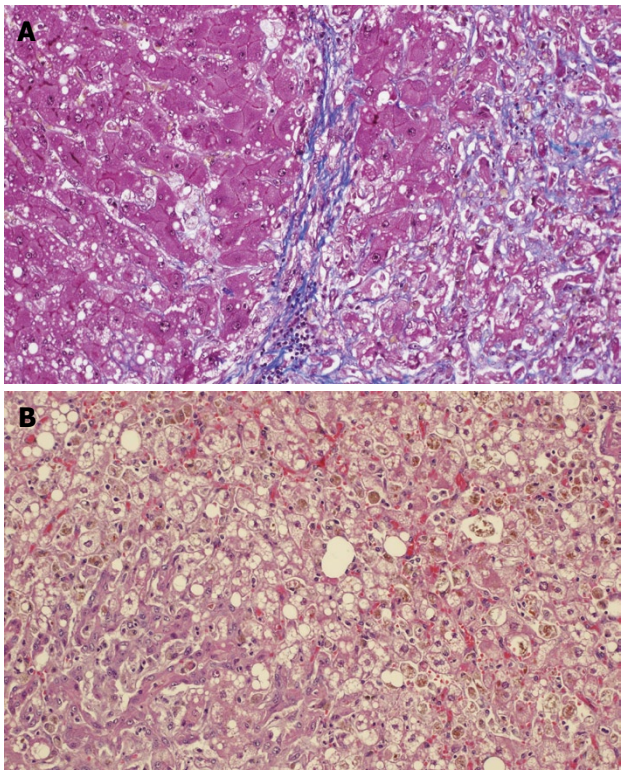

Figure 2 Liver necropsy. A: Stained with Mason trichromic; B: Stained with eosin-hematoxylin. FCH is characterized by marked hepatocellular swelling, lobular disarray and cholestasis, with only mild or no portal or lobular inflammation, combined with acute cholangiolitis and fibrosis surrounding the cholangioles.

DISCUSSION

To our knowledge, this is the first reported case of FCH after conventional cytotoxic chemotherapy treatment in a patient diagnosed with a solid tumor. There have only been 3 case reports published of FCH patients who had received conventional chemotherapy for hematological malignancies. FCH is a subtype of viral hepatitis in HVB-infected patients which is associated with extremely high mortality.

Degeneration of hepatocytes with minimal infiltration of inflammatory cells (which is clearly distinguishable from other fulminant hepatitis), extensive fibrosis and severe cholestasis are the most characteristic pathological findings. Liver parenchymatous changes include hepatocyte swelling and cholestasis with marked ductular reaction[13] (Figure 2A and B).

Although the ultimate physiopathological mechanism in this condition remains elusive[14], the extremely high levels of viral replication, the massive HBcAg and HBsAg expression in the liver and the non-significant inflammatory component suggest a direct HBV cytopathologic effect. The accumulation of viral antigens in the endoplasmic reticulum damages vital cell functions leading to cell death[13]. Although specific treatment protocols are lacking, a few case reports

have described a clinical benefit after administration of ganciclovir/foscarnet antiviral therapy[15] in these patients. Additionally, other reports describe the efficacy of lamivudine, a nucleoside analog reverse transcriptase inhibitor, in the treatment of chronic hepatitis[12] and in prophylaxis of chronic hepatitis and FCH[16]. Moreover, lamivudine has been recommended by some authors for the prophylaxis of HBV hepatitis in carrier subjects undergoing cytotoxic chemotherapy for lymphoid malignancies[17], although no data from clinical trials are available.

This case report suggests that viral analysis might be indicated in patients presenting with solid tumors before initiating intensive chemotherapy regimens. In addition, prophylactic lamivudine should be considered in HVB chronic infection carrier patients (HBsAg positive and/or HBcAb IgG positive) in this setting.

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CASE REPORT

Successful use of adalimumab for treating fistulizing Crohn's disease with pyoderma gangrenosum: Two birds with one stone

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Abstract

Crohn's disease (CD) is a chronic relapsing and remitting autoinflammatory disorder of the gastrointestinal tract that has many intestinal and extraintestinal complications. The purpose of treatment is long-term remission, reduction of complications, and improvement of patients' quality of life. In many cases, this can be quite challenging and it is necessary to have a well thought out management strategy. We present the case of a 38-year-old woman with fistulizing CD that manifested as diffuse abdominal pain and bloody diarrhea accompanied by arthralgia. In addition, there were ulcerative lesions surrounded by cutaneous inflammation and erythema on her extremities, indicative of pyoderma gangrenosum. The patient was treated with high doses of parenteral methylprednisolone without any improvement and was started on adalimumab. A positive response to adalimumab therapy was observed: after 2 mo of therapy, the ulcerative skin lesion healed completely and the enterogastric fistula was closed after 5 mo adalimumab treatment. Adalimumab might be a suitable initial as well as maintenance therapy in patients with complicated CD.

Key words: Adalimumab; Crohn's disease; Pyoderma gangrenosum

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INTRODUCTION

Crohn's disease (CD) is characterized by fissuring ulcers and segmental transmural inflammation of the gastrointestinal tract. The ileum is frequently involved in chronic inflammatory diseases; however, these can occur in any part of the digestive tract, from mouth to anus. Fistulas are the major and most common complications of the disease. The cumulative risk of any kind of fistula is 33% after 10 years and 50% after 20 years from the first appearance of the disease, as exemplified by a population-based study[1]. Although CD predominantly affects the gastrointestinal system, it is also associated with several extraintestinal manifestations. The most common extraintestinal disorders associated with inflammatory bowel disease (IBD) include dermatologic, ophthalmologic, musculoskeletal and hepatobiliary diseases; however, practically every organ system could be involved. These extraintestinal disorders can significantly contribute to morbidity and consequently impair the overall life quality of the patient considerably more than bowel-related symptoms.

Treatment is very complex and includes antibiotics and various immunomodulators. Surgery may be needed for therapy-refractory cases. The increasing number of advanced biological treatments for IBD offers new possibilities for the management of IBD associated with extraintestinal manifestations[2].

We report a case of successful adalimumab usage for CD complicated by enterocutaneous fistula, which also constituted an effective alternative treatment for pyoderma gangrenosum.

CASE REPORT

A 38-year-old woman presented with a 20-year history of CD, numerous complications and frequent relapses. Previously she had undergone several corrective gastrointestinal surgeries for perirectal fistulas. She carried a stoma since 1997 following left hemicolectomy and Hartmann's procedure. She continued to receive maintenance 5-aminosalycilate (mesalamine) and budesonide therapy. Corticosteroid therapy and combined antibiotic/antimycotic treatment were used intermittently for increased disease activity resulting in moderate clinical response. The patient was unable to tolerate azathioprine.

In February 2004, she developed erythema nodosum on her extremities, which was resolved by corticosteroid-antibiotic treatment. The patient was referred to our department for further examination and management in November 2004, when she developed asymmetric oligoarthritis that responded well to treatment with a corticosteroid and maintenance methotrexate, resulting in remission for 6 mo. In 2005, the patient was treated with intravenous pulse cyclophosphamide for moderate to severe disease activity as a rescue therapy.

The patient remained in remission for 6 mo. In January 2007, she was admitted to our hospital again because of increased weakness, mild ulcerative skin lesions, and abdominal pain with bloody diarrhea. Additionally, she had arthralgia but no fever. Her blood tests showed a white cell count of 8.97×10^9 cells/L, a platelet count of 289×10^9 cells/L, a C reactive protein level of 68.57 mg/L, a hematocrit of 36 and an increased erythrocyte sedimentation rate (64 mm/h). Immunologic evaluation did not show B-cell/immunoglobulin disorders or antibody positivity. Upon physical examination, she had some small, red papules on her extremities with ulcerations on their surface that were characterized by pyoderma gangrenosum (Figure 1). In addition, we also found diffuse abdominal tenderness and an abdominal mass located in the periumbilical region. Her urine and chest X-rays were normal. Abdominal computed tomography (CT) showed a moderately enlarged spleen as well as thickened and inflamed bowel walls that are characteristic of CD (Figure 2). The inflamed bowels were surrounded by fat stranding, and CT also showed multiple enterocolic fistulas. Upper endoscopic examination revealed an enterogastric fistula from the corpus of the stomach to the stoma. Surgical correction was not an option because the patient did not agree to surgery. The patient received infliximab (Remicade; Schering-Plough) therapy as well as maintenance methotrexate therapy but there was no significant improvement in her symptoms and in her test results.

Following a failed course of infliximab (because of lack of response), adalimumab (Humira; Abbott Laboratories) was proposed as an alternative treatment. Adalimumab was administered subcutaneously at a dose of 80 mg/wk for the first 2 wk followed by 40 mg/wk afterwards. In addition, the patient was treated for a limited period with high doses of parenteral methylprednisolone and a combined antibiotic treatment. After 2 mo of therapy, the skin ulcers were completely healed. Subjective clinical improvement could be seen after 3 mo of therapy. The patient did not experience any adverse effects. After 5 mo of therapy contrast radiography of the bowel passage showed significant improvement. A 3-4 cm long, narrow and blind fistula originating from the stomach could be seen but it was not connected to the bowel. Nevertheless, the duodenum and jejunum were moderately inflamed and the terminal ileum was intact without inflammatory signs.

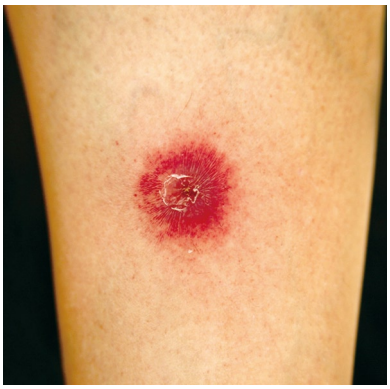

Figure 1 Small, red papules on the extremities with ulcerations.

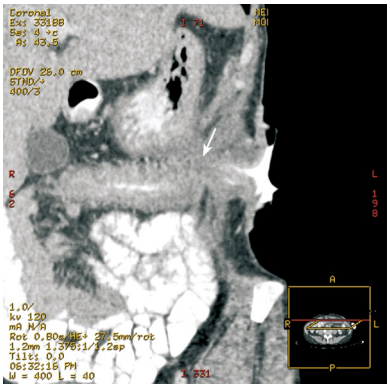

Figure 2 Enterogastric fistula from the corpus of the stomach to stoma on abdominal computed tomography (white arrow).

DISCUSSION

Up until now the use of adalimumab for fistulizing CD with extraintestinal manifestations has been reported in only a few cases. We reported a case of a woman with CD complicated by fistulas and pyoderma gangrenosum. The patient was successfully treated with adalimumab.

Extraintestinal manifestations of IBD can be diagnosed before, simultaneously with, or after the diagnosis of IBD is made. These symptoms occur in 21%-36% of IBD cases. It is important to distinguish complications of IBD from secondary diseases as they demand different and specialized therapies. The recognition of symptoms can be difficult since these extraintestinal symptoms can also be the primary manifestations of IBD[3,4].

The connection between different extraintestinal symptoms and IBD is unclear. The shared and unique epitopes in the human colon, eye, joint and biliary epithelium may suggest an immune mediated process.

Pyoderma gangrenosum is one of these inflamma-

tory cutaneous manifestations and it is independent of disease activity. This ulcerative cutaneous condition can either be associated with systemic inflammatory diseases (in at least 50% of cases) or it can occur alone. The diagnosis is made by excluding other causes of cutaneous ulcerations that are similar in appearance, including infection, malignancy, vasculitis, collagen vascular diseases, diabetes, and trauma. Treatment is relatively difficult. Currently, systemic immunosuppressants, often prednisone, are the mainstay of therapy. Long-term therapy with these agents is often required and can expose patients to possible adverse effects.

The treatment of IBD with extraintestinal manifestation has advanced in parallel to our increasing understanding of its pathomechanism[5-9]. One of the most well recognized proinflammatory mediators involved in the pathogenesis of IBD is tumor necrosis factor α (TNF-α). It is known that high levels of TNF-α have been associated with the development of intestinal inflammation in CD. The current evidence suggests that TNF-α blocking agents such as infliximab, adalimumab, and certolizumab pegol are effective maintenance therapy in CD. TNF-α blocking agents bind with TNF-α molecules thereby neutralizing the biological activity of TNF-α, resulting in the reduction of intestinal inflammation.

On other hand, treatment strategies for fistulizing CD are usually controversial. External fistulas are more responsive to medical therapy than internal fistulas in patients with CD. Combined treatment with antibiotics and immunomodulators may be a suitable initial therapy for CD patients with external fistulas. TNF-α blocking agents can also be used as an additional therapy in the treatment of corticosteroid dependent, refractory cases or in complicated extraintestinal manifestations[10].

This case report demonstrates the potential efficacy of adalimumab in the management of complicated fistulizing CD and/or pyoderma gangrenosum. Further studies into the use of adalimumab in this patient subgroup with CD are warranted.

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CASE REPORT

Scirrhous hepatocellular carcinoma displaying atypical findings on imaging studies

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Abstract

We describe a 15-mm scirrhous hepatocellular carcinoma (HCC) in a 60-year-old man with B-type cirrhosis. Ultrasound disclosed a 15-mm hypoechoic nodule in segment 7. Contrast-enhanced US revealed heterogeneous, not diffuse, hypervascularity in the early phase and a defect in the Kupffer phase. Contrast-enhanced computed tomography (CT) revealed a heterogeneous hypervascular nodule in the early phase and a low-density area in the late phase. Magnetic resonance imaging (MRI) revealed iso- to hypointensity at T1 and high intensity at T2-weighted sequences. Contrast-enhanced MRI also revealed a heterogeneous hypervascular nodule in the early phase and washout in the late phase. Super-paramagnetic iron oxide-MRI revealed a hyperintense nodule. CT during hepatic arteriography and CT during arterial portography revealed heterogeneous hyperattenuation and a perfusion defect, respectively. Based on these imaging findings the nodule was diagnosed as a mixed well-differentiated and moderately-differentiated HCC. Histologically, the nodule was moderately-differentiated HCC characterized by typical cytological and structural atypia with dense fibrosis. Immunohistochemically, the nodule was positive for heterochromatin protein 1 and alpha-smooth muscle actin, and negative for cytokeratin 19. From the above findings, the nodule was diagnosed as scirrhous HCC. Clinicians engaged in hepatology should exercise caution with suspected scirrhous HCC when imaging studies reveal atypical findings, as shown in our case on the basis of chronic liver disease.

Key words: Scirrhous hepatocellular carcinoma; Contrast-enhanced computed tomography; Contrast-enhanced magnetic resonance imaging; Contrast-enhanced ultrasound; Computed tomography during hepatic arteriography; Computed tomography during arterial portography; Heterogeneous hypervascularity

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INTRODUCTION

According to World Health Organization (WHO) classifications, hepatocellular carcinoma (HCC) with diffuse fibrosis is subclassified as scirrhous-type HCC (SHCC)[1]. Histologically, it is characterized by diffuse fibrosis along the sinusoid-like blood spaces with varying degrees of atrophy of tumor trabeculae. Preoperative images by computed tomography (CT) and magnetic resonance imaging (MRI) are, however,

often misdiagnosed as those of cholangiolocellular carcinoma (CCC), HCC-CCC, and metastatic carcinoma due to heterogeneous enhancement in the early phase and prolonged enhancement in the late phase attributed to abundant fibrous stroma. Moreover, imaging studies for the diagnosis of SHCC, such as contrast-enhanced ultrasound (US), CT during hepatic arteriography (CTA) and CT during arterial portography (CTAP) have so far not been described. Here, we present a case of moderately differentiated SHCC that histologically manifested as typical cytological and structural atypia with dense fibrosis, whereas imaging studies with contrast-enhanced CT, MRI, US, CTA and CTAP revealed a mixed well-differentiated and moderately-differentiated HCC.

CASE REPORT

A 60-year-old man with B-type liver cirrhosis was admitted in November 2007 for further examination of a 15-mm hypoechoic nodule in segment seven (S7). The patient had no history of alcohol, blood transfusion or drug abuse. On admission, physical examination showed no remarkable abnormalities. Hepatitis B virus was positive for surface antigen and for envelope antibody, and negative for envelope antigen (HBeAg). The amount of HBV deoxyribonucleic acid was less than 2.6 log copy/mL. Laboratory studies disclosed the following abnormal values: platelets $5.3 \times 10^4/\mu L$ (normal, 14-34), aspartate aminotransferase 44 IU/L (0-38), alkaline phosphokinase 864 IU/L (115-359), thymol turbidity 7.7 U (0-4), zinc sulfate turbidity test 14.8 U (2-12), and γ-globulin 29.3 g/dL (10.6-20.5). The levels of tumor markers were as follows: alpha-fetoprotein (AFP) 3.8 ng/mL (< 10), protein induced by vitamin K absence 71 mAU/mL (0-40), CA19-9 39.4 U/mL (0-37), and CEA 4.78 ng/mL (0-5).

US disclosed a 15-mm hypoechoic nodule in S7. Contrast-enhanced CT revealed a heterogeneous, not diffuse, hypervascular nodule in the early phase and a low-density area in the late phase (Figure 1A and B). MRI revealed iso- to hypointensity at T1 and high intensity at T2-weighted sequences. Contrast-enhanced MRI revealed a heterogeneous hypervascular nodule in the early phase and washout in the late phase (Figure 2A and B). Super-paramagnetic iron oxide-MRI revealed a hyperintense nodule. Contrast-enhanced US revealed heterogeneous hypervascularity in the early phase and a defect in the Kupffer phase (Figure 3A and B). CTA and CTAP revealed heterogeneous hyperattenuation and a perfusion defect, respectively (Figure 4). Based on these imaging findings, the nodule was diagnosed as a mixed well-differentiated and moderately differentiated HCC. Histologically, the nodule was moderately-differentiated HCC characterized by typical cytological and structural atypia with dense fibrosis (Figure 5A and B). Immunohistochemically, the nodule was positive for heterochromatin protein 1 and alpha-smooth muscle actin (α-SMA) (Figure 5C and D), and negative for cytokeratin 19 (CK19). From the above findings, the nodule was diagnosed as SHCC. We conducted radiofrequency ablation for the SHCC and the nodule was completely ablated. Local recurrence has not been observed over a period of 15 mo.

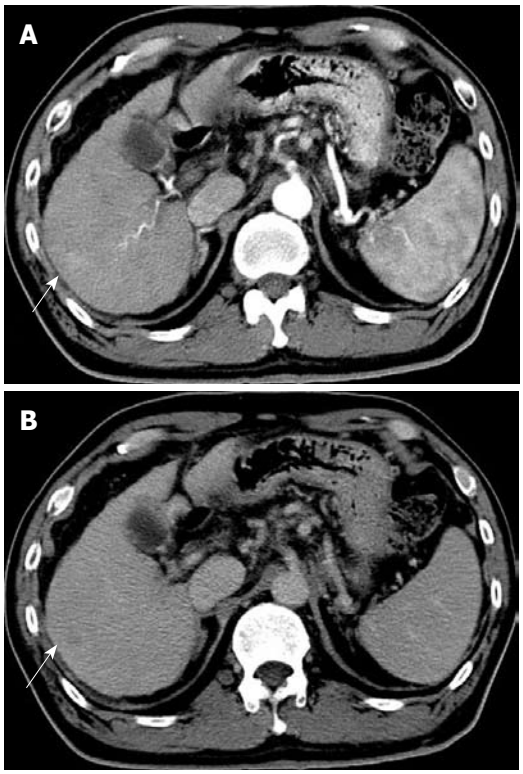

Figure 1 Contrast-enhanced CT. Heterogeneous, not diffuse, hypervascular nodule in the early phase (A) (arrow), and a low-density area in the late phase (B) (arrow).

DISCUSSION

The clinical background of SHCC is not significantly different from that of non-SHCC with regard to age, gender, positive rates to hepatitis viruses, AFP levels, Child-Pugh classification, and the stage of tumor-node-metastasis. In both morbidities, over 60% of cases are associated with chronic hepatitis rather than with liver cirrhosis. HCC patients with liver cirrhosis and liver dysfunction tend not to undergo surgery. In our case, resection was not carried out because of poor liver function attributed to liver cirrhosis.

With no clear pathological definition of SHCC, in particular a standard for the degree of the fibrosis for diagnosing the disease, its rate varies between 0.2% and 4.2%[2,3]. Regarding terminology, SHCC is often confused with "sclerosing hepatic carcinoma" that is used to designate a variety of tumors with sclerotic change and hypercalcemia arising in non-cirrhotic livers[4]. Sclerosing hepatic carcinoma does not, however, constitute a distinct histopathological entity; some of these tumors appear to be HCC, others CCC. Therefore, sclerosing hepatic carcinoma has been deleted from the WHO classification[1]. Kurogi *et al*[5] have defined SHCC as a tumor with diffuse fibrous changes in almost the entire area of the largest cross-section of the tumor and a mean fibrotic area of 39% compared with only 4.6%

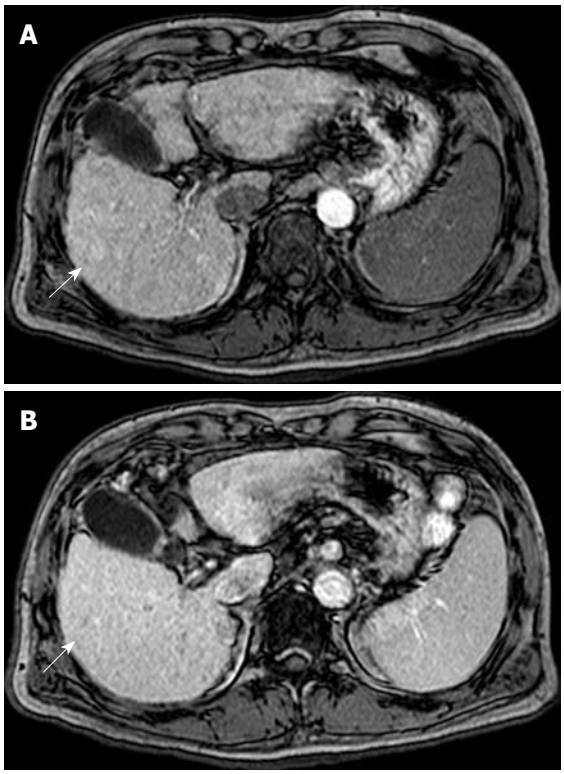

Figure 2 Contrast-enhanced MRI. A heterogeneous hypervascular nodule in the early phase (A) (arrow), and washout in the late phase (B) (arrow).

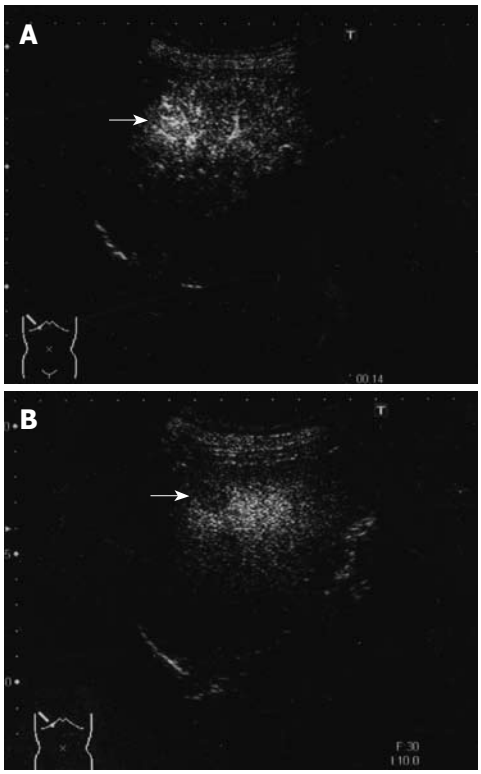

Figure 3 Contrast-enhanced US. Heterogeneous hypervascularity in the early phase (A) (arrow), and defect in the Kupffer phase (B) (arrow).

in non-SHCC.

SHCC is characterized by stellate fibrosis (84%), no encapsulation (absence of capsule) (100%), no necrosis and hemorrhage (100%), intratumoral portal tracts (80%), remarkable lymphocyte infiltration (84%), clear cell change (84%), and hyaline bodies (52%). The number of α-SMA-positive myofibroblast-like cells (activated stellate cells) in the tumor is about three times that in non-SHCC[5].

SHCC is occasionally misdiagnosed as fibrolamellar carcinoma (FLC) because of the presence of lamellar fibrosis. FLC is common in young adults and usually arises in the liver without any underlying chronic liver disease. Histologically, FLC is characterized by polyhedral, deeply eosinophilic neoplastic hepatocytes with round nuclei and distinct nucleoli, many of which contain intracytoplasmic hyaline globules and distinct pale bodies, and fibrosis arranged in a lamellar fashion around the neoplastic hepatocytes[6,7]. Conversely, although SHCC occasionally presents with lamellar fibrosis, the cancer cells being different from those of FLC, it is common in older patients with associated chronic hepatitis or liver cirrhosis[5]. Accordingly, it is not difficult to differentiate SHCC from FLC. In our case, the nodule was not diagnosed as FLC, clinically or histologically.

The US pattern was mostly hypoechoic, and contrast-enhanced CT and MRI revealed mostly heterogeneous hypervascularity in the early phase. The most characteristic feature of the imaging studies was prolonged enhancement in the late phase. Incidentally, imaging studies such as contrast-enhanced US, CTA and CTAP have, so far, not been described for use in the diagnosis of SHCC. Misdiagnosis by imaging studies is more frequent in SHCC than non-SHCC. Of 25 cases of SHCC, nine (36%) have been diagnosed as CCC, combined HCC-CCC, and metastatic carcinoma characterized by abundant fibrous stroma, the misdiagnosis being attributed to the prolonged enhancement of the tumor in the late phase and heterogeneous enhancement in the arterial phase on contrast-enhanced CT[5].

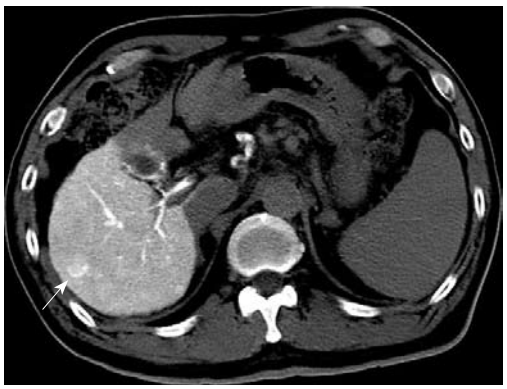

Figure 4 Heterogeneous hyperattenuation at CTA (arrow).

In our case, contrast-enhanced CT, MRI, US revealed heterogeneous hypervascularity in the early phase; the nodule was not misdiagnosed as CCC or HCC-CCC because the imaging findings showed no prolonged enhancement in the late phase. The nodule was misdiagnosed as well-differentiated and moderately-

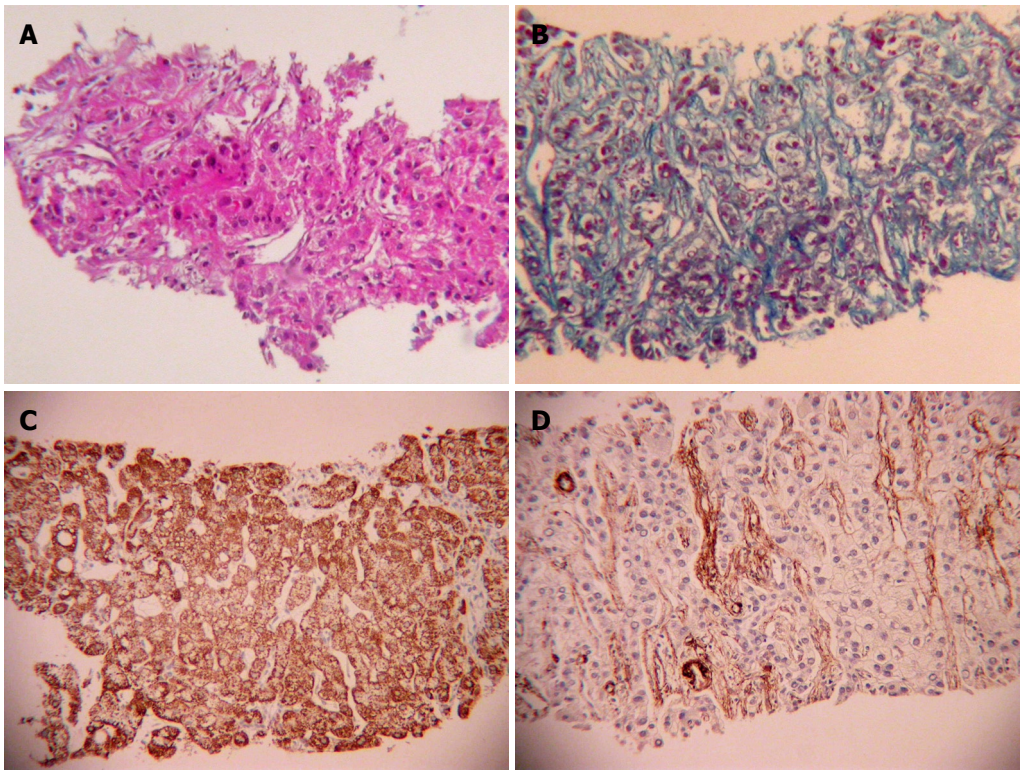

Figure 5 US-guided biopsy. Moderately-differentiated HCC characterized by typical cytological and structural atypia with dense fibrosis (HE stain) (A), (Mallory-Azan stain) (B). Positive for Hp1 (C) and α-SMA (D).

differentiated HCC on contrast-enhanced CT, MRI and US, which showed heterogeneous hypervascularity in the early phase and washout in the late phase; CTA and CTAP showed heterogeneous hypervascularity in the arterial and perfusion defect in the portal phases. Immunohistochemically, the nodule was negative for CK19 and, therefore, not CCC. Although contrast-enhanced US, CTA and CTAP did not indicate SHCC, these modalities are very effective in showing the heterogeneous vascular component, washout and perfusion defect of the nodule and contribute to precise diagnosis.

Clinicians engaged in hepatology should exercise caution with suspected SHCC when imaging studies reveal atypical findings, as shown in our case on the basis of chronic liver disease.

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Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

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Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
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The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
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Translation of the Cancer Genome

February 8-11, 2009
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Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

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February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
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Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
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World J Gastroenterol 2009 May 14; 15(18): 2302-2304
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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/eid.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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