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EDITORIAL

Risk for gastric neoplasias in patients with chronic atrophic gastritis: A critical reappraisal

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Abstract

Chronic atrophic gastritis (CAG) is an inflammatory condition characterized by the loss of gastric glandular structures which are replaced by connective tissue (non-metaplastic atrophy) or by glandular structures inappropriate for location (metaplastic atrophy). Epidemiological data suggest that CAG is associated with two different types of tumors: Intestinal-type gastric cancer (GC) and type I gastric carcinoid (T I GC). The pathophysiological mechanisms which lead to the development of these gastric tumors are different. It is accepted that a multistep process initiating from *Helicobacter pylori*-related chronic inflammation of the gastric mucosa progresses to CAG, intestinal metaplasia, dysplasia and, finally, leads to the development of GC. The T I GC is a gastrin-dependent tumor and the chronic elevation of gastrin, which is associated with CAG, stimulates the growth of enterochromaffin-like cells with their hyperplasia leading to the development of T I GC. Thus, several events occur in the gastric mucosa before the development of intestinal-type GC and/or T I GC and these take several years. Knowledge of CAG incidence from superficial gastritis, its prevalence in different clinical settings and possible risk factors associated with the progression of this condition to gastric neoplasias are important issues. This editorial intends to provide a brief review of the main studies regarding incidence and prevalence of CAG and risk factors for the development of gastric neoplasias.

Key words: Chronic atrophic gastritis; Gastric neoplasia; Intestinal-type gastric cancer; Type I gastric carcinoid; Prevalence; Incidence; Risk factors

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INTRODUCTION

Chronic atrophic gastritis (CAG) is an inflammatory condition characterized by the loss of gastric glandular structures which are replaced by connective tissue (non-metaplastic atrophy) or by glandular structures inappropriate for location (metaplastic atrophy)[1]. Epidemiological data suggest that CAG is associated with two different types of tumors: Intestinal-type gastric cancer (GC) and type I gastric carcinoid (T I GC). The pathophysiological mechanisms which lead to the development of these gastric tumors are different. It is accepted that a multistep process initiating from *Helicobacter pylori (H. pylori)*-related chronic inflammation of the gastric mucosa progresses to CAG, intestinal metaplasia, dysplasia, and finally leads to

the development of GC[2]. T I GC is a gastrin-dependent tumor and the chronic elevation of gastrin, which is associated with CAG, stimulates the growth of enterochromaffin-like (ECL) cells with their hyperplasia leading to the development of T I GC[3-5].

Considering that several events occur in the gastric mucosa before the development of GC and/or of T I GC, and that these events take several years, the knowledge of CAG incidence from superficial gastritis, its prevalence in different clinical settings and possible risk factors associated with the progression of this condition to gastric neoplasias are important issues.

EPIDEMIOLOGY OF CHRONIC ATROPHIC GASTRITIS

A recent systematic review was performed with the aim of evaluating the CAG incidence in patients free of CAG at moment of inclusion in the study[6]. From published studies, the authors selected only 14 follow-up studies in which CAG diagnosis was carefully made by histology (12 studies) or by serum pepsinogen (PG) levels (2 studies). The CAG incidence rates ranged from 0% to 10.9% per year. This wide CAG incidence range is explained by the particular settings in which the CAG diagnoses were made. In fact, the lowest incidence rates (0%) were found in patients with reflux esophagitis[7] and in patients successfully treated for *H. pylori* infection[8]. The highest incidence rate was observed in an older study conducted on patients who underwent vagotomy because of ulcer disease[9]. Regarding *H. pylori* infection, the CAG incidence rate was higher in *H. pylori*-positive patients than in *H. pylori*-negative ones[7,10-13] and the meta-analysis on the association between *H. pylori* infection and CAG incidence presented a rate ratio of 5 (95% CI: 3.1-8.3).

The prevalence of CAG was evaluated by serological screening using surrogate markers of gastric function (PG I or PG I/PG Ⅱ ratio) or by gastroscopy/histology. In the vast majority of cases, the serological and histological screenings were both made in a general population. Serological studies reported CAG prevalence rates between 3% and 7%, which were lower than those reported by histological ones. Studies on CAG prevalence subdivided on the basis of diagnostic tools used for CAG diagnosis (histology or serology) are shown in Table 1[14-23]. The observed differences between serological and histological studies could be explained by the fact that it is likely that symptomatic patients accepted more easily to undergo gastroscopy. Higher rates of CAG prevalence found in the Asian countries may be justified by the fact that these areas are at higher risk of GC and by the fact that the definition of CAG diagnosis may be different between Western and Asian countries. In studies reporting from Asian countries, CAG diagnosis included all atrophic lesions irrespective of the atrophy localization in the gastric mucosa (antrum and/or corpus); in the vast majority of the studies conducted in Western countries, CAG diagnosis included only patients with a corpus atrophic involvement such as corpus-atrophic gastritis or a multifocal atrophic gastritis (i.e., patchy areas of atrophic-metaplastic changes in the antral and corpus mucosa), because it is maintained that only corpus atrophic changes can lead to the development of gastric cancer.

ATROPHIC GASTRITIS AND GASTRIC CANCER

Nowadays, GC represents one of the most challenging tumors due to the fact that its diagnosis is often late and, in the advanced stage, the therapeutic options are scarce with consequent high rate of mortality[24]. In fact, although a reduction of global incidence for this neoplasm is reported, it remains the second cause of cancer-related death. The knowledge of precursor lesions for the development of intestinal-type GC could contribute to anticipating GC diagnosis at an early stage when surgery or chemotherapy offers a better prognosis. Several studies have estimated the risk of GC in patients with CAG[25-33]. Although the vast majority of these were performed on small numbers of patients and were based on older histological classifications, the progression rate of CAG to GC fluctuates from 0% to 10% with annual incidence (person-year) lower than 1% (Table 2). It is interesting to observe that, although the incidence rate of CAG in patients with superficial gastritis is higher in populations with higher risk of GC (Table 1), the progression rate of CAG towards GC is similar irrespective of different geographic areas.

Some studies have attempted to identify risk factors linked with the progression of precancerous lesions (CAG or intestinal metaplasia) towards GC to select those patients who should undergo endoscopic surveillance.

Age

Age has been identified as a possible risk factor in several studies. In the study by Leung *et al*[40], *H. pylori*-positive patients with intestinal metaplasia were followed up for 5 years to evaluate the progression or the improvement of histological lesions after *H. pylori* eradication treatment compared with placebo. At multivariate analysis, the presence of age > 45 years showed an approximate two-fold increased risk of progression of intestinal metaplasia compared to younger subjects[40]. This same age limit had already been identified in a screening survey performed on 3386 subjects from a rural Chinese population that showed an approximate three-fold increased risk of progression to GC[28]. In a large cohort study, increasing age at initial diagnosis was associated with higher hazard ratio (HR) for the progression to GC (for age > 55 years, HR > 2.38)[32]. In a recent work, patients with CAG who were aged > 50 years at the moment of initial diagnosis presented HR = 8.8 for the progression to gastric neoplastic lesions[33].

Pernicious anemia

Although the vast majority of the older studies on CAG

Table 1 Prevalence of chronic atrophic gastritis

Author	Year	Country	Study type	Patients	Age (yr)	CAG (%)
Serology						
Sipponen *et al*[14]	2003	Finland	General population	12 252 (men)	51-65	5.2
Green *et al*[15]	2005	New Zealand	General population	466	> 65	6.7
Weck *et al*[16]	2007	Germany	General population	9444	50-74	6
Telaranta-Keerle *et al*[17]	2010	Finland	General population	4256	18-92	3.5
Histology						
Oksanen *et al*[18]	2000	Finland	Endoscopic cohort	207	19-83	13[1]
Borch *et al*[19]	2000	Sweden	General Population	501	35-85	9.4[2]
Asaka *et al*[20]	2001	Japan	General Population	2455	< 20 to > 70	55.5[3]
Redéen *et al*[21]	2003	Sweden	General Population	488	37-85	9
Storskrubb *et al*[22]	2008	Sweden	General Population	976	20-80	6.6[4]
Zou *et al*[23]	2011	China	General Population	1022	18-80	63.8[3]

[1]This percentage refers to patients (n = 27) with atrophic body gastritis; [2]this percentage refers to patients (n = 47) with atrophic pangastritis and corpus- predominant (gastritis; [3]these percentages included chronic atrophic gastritis (CAG) diagnosis irrespective of the atrophy localization in the gastric mucosa (antrum and/or corpus); [4]this percentage refers to patients (n = 54) with multifocal atrophic gastritis and atrophic corpus- limited gastritis.

Table 2 Incidence of gastric cancer in patients with chronic atrophic gastritis or pernicious anemia

Author	Year	Country	Study type	Patients	Age, median or range (yr)	GC	Annual incidence of GC, person-year (%)
Patients with chronic atrophic gastritis							
Walker *et al*[25]	1971	Australia	Retrospective	40	40-64	4 (10)	0.6
Ectors *et al*[26]	1986	United Kingdom	Retrospective	225	-	3 (1.3)	0.1
Tatsuta *et al*[27]	1993	Japan	Retrospective	654	-	22 (3.4)	0.2
You *et al*[28]	1999	China	Prospective	2082[1]	35-64	19 (0.9)	0.2
Whiting *et al*[29]	2002	United Kingdom	Prospective	1042	> 40	12 (11.5)	1.1
Dinis-Ribeiro *et al*[30]	2004	Portugal	Retrospective	1771	-	4 (2.2)	0.7
Lahner *et al*[31]	2005	Italy	Prospective	106	22-74	1 (0.9)	0.1
de Vries *et al*[32]	2008	Netherlands	Retrospective	84 072[2]	65.7	1035 (1.2)	0.2
Vannella *et al*[33]	2010	Italy	Retrospective	300	18-78	3 (1)	0.2
Patients with pernicious anemia							
Borch *et al*[34]	1986	Sweden	Prospective	61	-	0	0
Kokkola *et al*[35]	1998	Finland	Prospective	62	20-73	2 (3.2)	1.10
Sjöblom *et al*[36]	1993	Finland	Prospective	56	27-78	2 (3.5)	1.20
Armbrecht *et al*[37]	1990	United Kingdom	Prospective	27	26-81	0	0
Bresky *et al*[38]	2003	Spain	Prospective	68	-	0	0
Ye *et al*[39]	2003	Sweden	Retrospective	21 265	74.3	177 (0.8)	0.10
Vannella *et al*[33]	2010	Italy	Retrospective	129	23-74	2 (1.5)	0.30

[1]This number refers to biopsies taken in 144 patients and includes chronic atrophic gastritis (CAG) with type Ⅰ, Ⅱ, Ⅲ intestinal metaplasia; [2]this number refers to CAG patients with or without intestinal metaplasia. GC: Gastric cancer.

included patients with pernicious anemia, the risk of GC in this particular clinical setting seems to be generally low (Table 2). In fact, this clinical condition is often associated with corpus-restricted gastritis and, as a consequence, with less extensive atrophy in the gastric mucosa. In a recent study, the presence of atrophic pangastritis increased the risk of progression to gastric neoplastic lesions by 4.5 times, in keeping with previous works[33,41,42]. The apparent contrast between older and more recent works about pernicious anemia can be explained by the difficulty in comparing studies with methodological differences linked to adopted gastritis classification or small number series. It is interesting to underline the fact that studies on the relationship between pernicious anemia and GC are lacking in Asian countries where the risk of GC is higher, thus it remains to be established whether pernicious anemia has low prevalence in the Asian geographic area or if this condition is overlooked.

Intestinal metaplasia

Parallel with more extensive atrophy in the gastric mucosa, the extensive replacement of this by intestinal metaplasia is considered a hallmark of severity of CAG. In the literature, the intestinal metaplasia extension was widely related to a higher risk of GC[32,33,40,42]. In particular, type Ⅲ intestinal metaplasia was associated with an increased risk of GC in some studies[43,44], but subsequent studies showed conflicting findings[45,46], thus the clinical utility of different subtyping of intestinal metaplasia is limited.

Helicobacter pylori

The role of *H. pylori* infection in progression from CAG

to GC is controversial. In the Leung study, *H. pylori*-positive patients who had not undergone eradication therapy had a progression rate of intestinal metaplasia higher than cured patients[40]. However, in this study, the vast majority of patients had only a superficial gastritis at baseline and, after 5 years of follow-up, the rate of patients with intestinal metaplasia increased significantly. It is maintained that the effect of eradication therapy on the progression to GC in patients with precancerous lesions is limited. A previous large prospective study demonstrated that *H. pylori* eradication may be beneficial in arresting the progression to GC only in patients without CAG or intestinal metaplasia[47]. Two recent meta-analyses showed a beneficial long-term effect of *H. pylori* eradication therapy on atrophic gastritis, but not on intestinal metaplasia[48,49]. Up till now, although the possibility of histological improvement of CAG is accepted after *H. pylori* cure, the efficacy of *H. pylori* eradication in reducing GC incidence needs to be demonstrated.

ATROPHIC GASTRITIS AND TYPE I GASTRIC NEUROENDOCRINE TUMOR

T I GC derives from ECL cells which are localized in the gastric fundus and corpus. ECL cells are specialized in the secretion of histamine that, in turn, stimulates acid secretion by parietal cells[50]. Gastric carcinoids have been classified into three subgroups, type I to type Ⅲ, with different outcomes[51-53]. Type I lesions are associated with atrophic gastritis and constitute up to 80% of all gastric carcinoids[54]. Gastrin, released by G-cells in the gastric antrum, stimulates the release of histamine and produces trophic effects upon ECL cells[3]. In CAG, the loss of appropriate glands in the body leads to achlorhydria, and the consequent chronic hypergastrinemia stimulates ECL hyperplasia and sometimes the development of T I GC[4,5].

The prevalence rate of T I GC in patients with CAG is reported to be between 1% and 12.5% in different studies[36,37,55-58]. The wide range of the prevalence rates of T I GC among several studies can be explained by different settings where patients were selected, such as type of hospital (secondary, tertiary center) or symptoms/signs of presentation. CAG can have a wide range of clinical presentations such as dyspepsia, iron deficiency anemia or pernicious anemia[59]. In particular, in a recent observational study in which the T I GC incidence and prevalence were evaluated, pernicious anemia was present in almost 50% of patients, while previous studies included exclusively patients with this condition[60].

Long-term observational studies assessing incidence of T I GC in CAG patients are scarce[35,56,61]. We recently followed up a cohort of CAG patients for 1463 person-years reporting an annual incidence rate (person-year) for T I GC of 0.4%[60]. An old study by Kokkola *et al*[35] reported an annual incidence of 2%, observing 8 new cases of T I GC in 416 patient-years. Sjöblom *et al*[61] studied 196 patients with pernicious anemia and after 1397 patient-years, 2 new cases of T I GC were reported in hospital registries among the initial group of patients. This figure should correspond to an annual incidence rate of 0.1%, but in this study only 70 patients (35.7%) underwent gastroscopy and the incidence rate can only be obtained indirectly. Furthermore, although there are small fluctuations in the reported incidence rates, only a small group of CAG patients develop T I GC showing that factors other than gastrin are necessary for the progression of ECL cells to T I GC.

Few studies have attempted to identify risk factors associated with the development of T I GC. In a recent work, we found higher baseline levels of gastrin and chromogranin A in CAG patients with T I GC compared to those without T I GC. However, all patients with CAG present high plasma values of chromogranin A[62] and gastrin, thus these markers have limited clinical utility because of low specificity[63].

An accepted risk factor for T I GC is the presence of ECL dysplasia, which is often associated with T I GC. This lesion is considered as the true gastric carcinoid precursor lesion and it can represent the sign of a concomitant carcinoid lesion[56,64]. CAG patients with a diagnosis of ECL cell dysplasia could benefit from a shorter endoscopic follow-up time to exclude concomitant T I GC lesions or to identify newly arisen lesions in the gastric mucosa.

Although T I GC lesions can also be present on flat mucosa, in the vast majority of cases they are associated with the presence of body polyps. In CAG patients, hyperplastic or adenomatous polyps are very common; however, the presence of body polyps increases the risk of having a T I GC[60]. Unfortunately, no feature of endoscopic appearance of the gastric polyps (size, number, sessile/pedunculated presentation) seems useful to differentiate histology of polyps, thus all polyps should be removed and histologically examined[65,66].

CONCLUSION

The risk of development of GC or T I GC appears higher in CAG patients with respect to the general population. In geographic areas with low risk of GC, a surveillance program for all CAG patients may be not cost-effective considering that the vast majority of CAG patients will not develop a gastric neoplasm[67]. A subset of CAG patients at higher risk for GC should be identified allowing the selection of those CAG patients in whom gastroscopic/histologic surveillance may be warranted. Recently, an international consensus developed evidence-based guidelines on the management of precancerous conditions and lesions of the stomach, recommending an endoscopic surveillance every 3 years after diagnosis in all patients with extensive atrophy and/or intestinal metaplasia in the antrum and corpus[68]. New systems for histopathological staging (OLGA, OLGIM) have been developed with the aim of combining pathological findings with the risk of GC for the patient and to iden-

tify a subgroup of those at higher risk[69,70]. The OLGA system includes gastritis grading and staging[69]. Grading measures the severity of acute and chronic inflammatory infiltrate in the antrum and body. Staging refers to the extent of atrophy with or without intestinal metaplasia. The OLGIM system is based on intestinal metaplasia which is considered a more reproducible histopathological diagnosis with respect to atrophy diagnosis. Further studies are necessary to validate these new classifications and to establish their real clinical value. Regarding T I GC, although risk factors for its development have not been identified, ENETS guidelines suggest an endoscopic follow-up every 6-12 mo after T I GC diagnosis. This interval allows the identification of recurrent lesions or new lesions (incidence-case) at an early stage when they can easily be removed by polypectomy without complications[71]. This approach seems safe for T I GC, a neoplasm with an excellent outcome[60,72].

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TOPIC HIGHLIGHT

Luis Bujanda, Professor, *Series Editor*

Mouse models of pancreatic cancer

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Abstract

Pancreatic cancer is one of the most lethal of human malignancies ranking 4th among cancer-related death in the western world and in the United States, and potent therapeutic options are lacking. Although during the last few years there have been important advances in the understanding of the molecular events responsible for the development of pancreatic cancer, currently specific mechanisms of treatment resistance remain poorly understood and new effective systemic drugs need to be developed and probed. *In vivo* models to study pancreatic cancer and approach this issue remain limited and present different molecular features that must be considered in the studies depending on the purpose to fit special research themes. In the last few years, several genetically engineered mouse models of pancreatic exocrine neoplasia have been developed. These models mimic the disease as they reproduce genetic alterations implicated in the progression of pancreatic cancer. Genetic alterations such as activating mutations in KRas, or TGFb and/or inactivation of tumoral suppressors such as p53, INK4A/ARF BRCA2 and Smad4 are the most common drivers to pancreatic carcinogenesis and have been used to create transgenic mice. These mouse models have a spectrum of pathologic changes, from pancreatic intraepithelial neoplasia to lesions that progress histologically culminating in fully invasive and metastatic disease and represent the most useful preclinical model system. These models can characterize the cellular and molecular pathology of pancreatic neoplasia and cancer and constitute the best tool to investigate new therapeutic approaches, chemopreventive and/or anticancer treatments. Here, we review and update the current mouse models that reproduce different stages of human pancreatic ductal adenocarcinoma and will have clinical relevance in future pancreatic cancer developments.

Key words: K-Ras; Mouse models; Transgenic; Pancreatic cancer; Xenografts

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INTRODUCTION

Infiltrating ductal adenocarcinoma of the pancreas (PDAC) accounts for over 85% of all pancreatic malignancies and has a poor prognosis as less than 5% of patients survive 5 years after diagnosis with a median survival period of 4-6 mo[1-3]. During the last few years there have been important advances to better understand the molecular mechanisms regulating the development of PDAC[4,5]. However, prog-

ress in prevention, early diagnosis and treatment needs major advances[6].

Some of the recent advances have been possible by employing mouse models which have provided an important model system to better understand the molecular mechanism underlying pancreatic cancer. However, in stark contrast to the successful murine models of most common human tumors, the generation and use of appropriate mouse models of pancreatic cancer has remained an area of significant frustration and not always well established. Currently, there are several different genetically modified mouse tumors and xenograft models available that offer the possibility of experimental and preclinical model systems to evaluate different strategies for targeting this disease, early detection, chemoprevention, treatment and finally improve the outcome for pancreatic cancer patients[7].

These models use a variety of approaches to target the expression of mutant or endogenous specific genes and as a result they develop a broad spectrum of pathologic changes, some of them mimic human disease while others are not equivalent to human pancreatic neoplasia. According to the cancer progression model postulated by Fearon and Volgestein[8] in 1990, at least 4-5 genetic events are required for the progression from normal epithelium to carcinoma. Since, the genetic basis of pancreatic ductal adenocarcinoma was revealed, with activation of Kras and inactivation of the p16INK4a, p53 and Smad4 tumor suppressors[9], several mouse models of invasive pancreatic cancer have been developed and modified. Also, regarding the role of pancreatic intraepithelial neoplasia (PanIN) as a direct noninvasive neoplastic precursor to human pancreatic cancer[10], different mouse models are currently available, some of these models reproduce only PanIN lesions and others progress to invasive pancreatic carcinoma. Most of these models were previously presented and evaluated at the International Workshop sponsored by the National Cancer Institute and the University of Pennsylvania in 2004. Twelve genetically engineered mouse models were included and have been considered models for the study of pancreatic disease including PanINs and carcinomas[11-18]. Since then, several new models have been introduced in the basic and translational research fields and previous models have been re-evaluated. Here, we will focus only on pancreatic cancer mouse models as PanIN lesions are considered preinvasive.

Since an activating mutation of the Kras oncogene is the most frequent genetic alteration associated with pancreatic cancer, having been identified in up to 90% of all pancreatic adenocarcinomas[19-21], most of the genetically engineered mouse models are based on the Kras oncogene. As mice expressing mutant Kras develop early and advanced forms of the most common pancreatic cancers in humans, these Kras-based models provide preclinical model systems to analyze the molecular biology of this disease and measure the benefit of new therapies[7,22].

In these review, we update and describe the most common genetically engineered mouse and xenograft models of PDAC that could be useful for assessing the role of genes and pathways, environmental conditions, co-morbidities and response to new adjuvant, neoadjuvant and anti-metastatic therapies.

TRANSGENIC MOUSE MODELS

As Kras mutations are not sufficient to induce progression to the invasive stage of pancreatic adenocarcinoma, different transgenes have been used to generate combined models that progress to invasive PDAC and metastatic disease.

The common genetically engineered models are based on Kras mutations and also include PDX-1-Cre/Lox-Stop-Lox (LSL)-Kras or p48/LSL-Kras mice which have been modified with deletions or mutations of Ink4[23], p53[24], Mist[25], Smad4[26] or TGFβ[27] (Table 1).

These Kras-mutated models can be induced using inducible alleles of Cre recombinase, such as estrogen receptor-Cre fusion genes (*CreER* or *CreERT*) and cycline-responsive Cre expression alleles (TRE-Cre) which are temporally expressed and initiate the expression in adult pancreata reflecting the somatic mutation as it occur in humans[28,29]. Also, some models that only develop PanIN lesions are available as Ela-LSL- $Kras^{G12D}$[12], Nestin-Cre, LSL-$Kras^{G12D}$[30], PDX-1-CRE^{ERT}, LSL-$Kras^{G12D}$, R26Notch^{NIC}[31] and PDX-1-CRE, LSL-$Kras^{G12D}$, Tif1$\gamma^{flox/flox}$[32], however, these are not the purpose of our review.

PDX1-Cre, LSL-$Kras^{G12D}$ and P48$^{+/-Cre}$, LSL-$Kras^{G12D}$ transgenic model

After different studies identified PDX-1 and p48 as critical transcription factors in the developmental program of the pancreas[21,33], these genes have been used in almost all transgenic mouse models to study pancreatic cancer. It is well known that the first identifiable pancreatic progenitor cell in the pancreas arises in the dorsal and ventral endoderm at embryonic day 8 in the fetal mouse: expression of PDX-1 occurs around E8.5[34] and P48 is expressed slightly later and is required to commit cells to a pancreatic fate[35].

In addition, Ptf1a, a component of the pancreas transcription factor 1 complex (Ptf1) which plays an important role in mammalian pancreatic development has been used in some mouse models. Pdf1a determines whether cells allocated to the pancreatic buds continue towards pancreatic organogenesis or revert to duodenal fates[36,37]. To target the expression of oncogenic Kras in pancreatic progenitor cells, a conditionally expressed allele was constructed as previously described by Jackson *et al*[38].

Briefly, the targeting vector contains genetic elements inhibiting transcription and translation flanked by functional LoxP sites. This Lox-Stop-Lox (LSL) construct was inserted into the mouse genomic Kras locus upstream of locus 1 to contain G-A transition in codon 12 (G12D). This transition mutation results in a glycine to aspartic acid substitution in the expressed protein that activates constitutive downstream signaling of Ras effector pathways and is one of the most common mutations found in human pancreatic tumors.

Table 1 Mouse models of pancreatic adenocarcinoma

Genotype (reference)	Time of expression	Time to tumor development (mo)	Pancreatic cancer phenotype	Survival (mo)
PDX-1-Cre; LSL-Kras^{G12D}[39]	E8.5	6	PDAC; penetrant PanIN; age dependent increase severity; occasionally PDAC with long latency	16
P48$^{+/-Cre}$; LSL-Kras^{G12D}[39]	E9.5	8	PDAC; penetrant PanIN; age dependent increase severity; occasionally PDAC with long latency	16
PDX-1-Cre; LSL-Kras^{G12D}; LSL-Trp53$^{R172H/-}$[24]	E8.5	2-3	PDAC Accelerated PanIN; well differentiated PDCA	5-6
Mist1$^{KrasG12D/+}$[25]	E10.5	2	Accelerated development of acinar-derived PanIN; mixed subtypes pancreatic cancer	10.8
KPCB$^{wt/wt}$[42]	E8.5	2-3	PDAC	5.6
KPCB$^{Tr/wt}$[42]	E8.5	3	PDAC	4.8
KPCB$^{Tr/\Delta 11}$[42]	E8.5	1.5	PDAC; mixed	2.8
CKB$^{wt/\Delta 11}$[41]	E8.5	6	PDAC	12
CKB$^{wt/wt}$[41]	E8.5	6	PDAC	13.5
CPB$^{\Delta 11/\Delta 11}$[41]	E8.5	3-5	PDAC; mixed	10
Pdx1-Cre; Kras^{G12D} Ink4a/Arf$^{flox/flox}$[23]	E8.5	2	PDAC; accelerated development of PanIN; poorly differentiated PDAC	2-3
Pdx1-Cre; Kras^{G12D} Smad4$^{flox/flox}$[55]	E8.5	2-3	IPMN; PDAC	2-6
Ptf1a$^{cre/+}$; LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/flox}$[27]	E9.5	1	PDAC; accelerated PanIN; PDAC development	2

PDAC: Ductal adenocarcinoma of the pancreas; PanIN: Pancreatic intraepithelial neoplasia; IPMN: Intraductal papillary mucinous neoplasia.

Hingorani *et al*[39] developed a mouse model expressing a Cre-activated Kras^{G12D} allele inserted into the endogenous Kras locus, and these mice were crossed with mice expressing Cre recombinase in pancreatic tissue, either by virtue of a PDX-1 promoter-driven transgene or by Cre knockin at the Ptf1-p48 locus. Prior lineage studies suggest that both of these lines express Cre in a common endocrine/exocrine precursor cell during development, while expression in adults is retained in mature islet cells in the case of PDX-1-Cre transgenics and in mature acinar cells in the case of the Ptf1-p48+/Cre knockin[35].

The subsequent recombination resulted in interbreeding LSL-Kras^{G12D} mice with animals that express Cre recombinase from the pancreatic-specific promoters PDX-1 or P48 is a heterozygous mutant condition (KRAS$^{+/G12D}$). Note that only genomic DNA isolated from pancreata and not from tails evidence the recombination. The mutant mice PDX-1-Cre, LSL-Kras^{G12D} and P48$^{+/-Cre}$, LSL-Kras^{G12D} have increased Kras oncogenic protein and their pancreata are larger than their wild type littermate controls.

The pancreata of compound mutant mice develop ductal lesions identical to all three stages of human PanINs. PanIN-1A lesions are observed in compound mutant mice as young as 2 wk old. As the mice age, higher-grade PanINS were observed with increasing frequency and in many of the older mice, the pancreata contained extensive ductal lesions and the acinar parenchyma was replaced by stromal or desmoplastic fibroblasts and inflammatory cells. This fibroinflammatory reaction is highly reminiscent of that seen in human pancreatic cancers. PanIN lesions show evidence of histologic progression and it has been demonstrated that these PanINs activate quiescent pathways such as Notch. These mice have increased Hes1 and Cox2, components of the prostaglandin pathway involved in the inflammatory response and increased matrix metalloproteinase-7. Finally, at low frequency these mice progress to invasive and metastatic ductal adenocarcinoma within one year. In these mice, profuse hemorrhagic ascites was noted, the pancreas was large, firm and fibrotic and nodular densities were observed in liver, diaphragm, pleural surfaces and adrenal cortex.

This model developed by Hingorani *et al*[39] shows progressive PanIN lesions and low-frequency progression to invasive and metastatic adenocarcinoma following activation of oncogenic K-Ras in mouse pancreas. The physiopathology and the sites of metastases observed in these mice are precisely found in human pancreatic ductal adenocarcinoma and further underscore the applicability of this model to study the human disease.

PDX-1-Cre, LSL-Kras^{G12D}, LSL-Trp53$^{R172H/-}$ transgenic model

This mouse model was generated based on the previously described PDX-1-Cre, LSL-Kras^{G12D} mouse. Using similar methods, Hingorani *et al*[24] generated a conditionally expressed point mutant allele of the Li-Fraumeni human ortholog, Trp53^{R175H}[40]. Activation of both the Kras^{G12D} and the Trp53^{R172H} alleles occurs in tissue progenitor cells of the developing mouse pancreas through interbreeding with PDX-1-Cre transgenic animals. The presence of each rearranged, activated allele can be detected in the pancreata but not in tails. Thus, tissues not expressing Cre recombinase (non-pancreatic tissue) remain functionally heterozygous for these loci.

Four to six weeks old mice PDX-1-Cre, LSL-Kras^{G12D}, LSL-Trp53$^{R172H/-}$ present early PanIN lesions similar to what it is observed in single PDX-1-Cre, LSL-Kras^{G12D} mice. A significant disease burden is observed in animals by ten weeks of age at the earliest and the full spectrum of preinvasive lesions is apparent. Histological analyses reveal a predominant moderately well-differentiated to well-differentiated morphology organized as is observed

in the human disease. The carcinomas express CK19 and frequently contain mucin. Metastasis to the liver and lungs are similar to the pancreatic primaries. Finally, PDX-1-Cre, LSL-Kras^{G12D}, LSL-Trp53$^{R172H/-}$ mice have dramatically shortened median survival of approximately 5 mo, significantly less than wild type, PDX-1-Cre, LSL-Trp53$^{R172H/-}$ and PDX-1-Cre, LSL-Kras^{G12D}.

The triple mutant mice succumb earlier than PDX-1-Cre, LSL-Kras^{G12D} animals which spontaneously develop PDA with a proscribed latency after manifesting preinvasive neoplasia. These triple mutant animals develop cachexia, abdominal distension, and hemorrhagic ascites. They also present metastasis in the liver, diaphragm and adrenals and all of them die before 12 mo.

PDX-1-Cre, Brca2^{F11}, LSL-Kras^{G12D}, Trp53 F2-10 transgenic model

This transgenic mouse is a conditional Brca2^{F11}, LSL-Kras^{G12D}, Trp53 F2-10 and PDX-1-Cre and has been used as a model of pancreatic cancer, although the role of Brca2 in pancreatic cancer development is still unclear[41,42]. Brca2 plays a key role in the maintenance of genomic integrity, particularly through regulation of DNA repair by homologous recombination repair[43], a process that is also controlled by another tumor suppressor protein, Brca1[44]. However, the significance of Brca2 in pancreatic cancer is not clear[45].

While Rowley *et al*[41] demonstrated that the inactivation of Brca2 promotes Trp53-associated but inhibits Kras^{G12D}-dependent pancreatic cancer development in mice, Skoulidis *et al*[42] showed that Brca2 heterozygosity promotes Kras^{G12D}-driven carcinogenesis in the murine model of familial pancreatic cancer. In this model, the mouse expressed a functional wild type *Brca2* gene, in which exon 11 of Brca2 is flanked by loxP sites (B2^{F11}). Conditional rearrangement of this allele in the developing pancreas in response to PDX-1-Cre expression results in the deletion of Brca2 exon 11, and the generation of a functionally null Brca2 allele (B2$^{\Delta 11}$). These authors crossed CB2$^{\Delta 11/\Delta 11}$ mice with conditional Trp53F2-10/F2-10 (P) mice, in which exons 2 and 10 are flanked by loxP sites to generate Trp52 null CPB2$^{\Delta 11/\Delta 11}$, CPB2$^{wt/\Delta 11}$ and CPB2$^{wt/wt}$ mice.

CPB2$^{\Delta 11/\Delta 11}$ mice develop pancreatic cancer at high frequency and their median survival is 300 d, showing substantially reduced pancreatic cancer-free survival relative to CB2$^{wt/\Delta 11}$. However, in contrast, CB2$^{\Delta 11/\Delta 11}$, CB2$^{wt/\Delta 11}$ and CB2$^{wt/\Delta 11}$ mice expressing wild type Trp53 alleles failed to develop pancreatic cancer.

This mouse model shows that the inactivation of Brca2 alone does not promote pancreatic cancer, but the disruption of Trp53 signaling in combination with the inactivation of Brca2 promotes pancreatic cancer formation. CPB2$^{\Delta 11/\Delta 11}$ mice display severe acinar cell dysplasia and a reduced number of islets. The pancreas is atrophic with acini replaced by mature adipose tissue, inflammatory infiltrates and little evidence of fibrosis. In contrast, in CPB2$^{wt/\Delta 11}$ and CPB2$^{wt/wt}$ mice the dysplasia, atrophy and chronic inflammatory infiltrate is less severe and frequent[41]. The mouse model combining Brca2^{F11} and LSL-Ras^{G12D} (K) shows that CKB2$^{\Delta 11/\Delta 11}$, CKB2$^{\Delta 11/\Delta 11}$ and CKB2$^{wt/wt}$ mice display normal development although CKB2$^{wt/\Delta 11}$ and CKB2$^{wt/wt}$ present PanINs and metaplastic lesions at 8 mo but not CKB2$^{\Delta 11/\Delta 11}$. This mouse model showed that the loss of Brca2 tumor suppressor inhibits the development of premalignant lesions and pancreatic tumors that are induced by activated Kras. Only 13% of CKB2$^{\Delta 11/\Delta 11}$ mice develop tumors, whereas 66% of CKB2$^{wt/\Delta 11}$ and 61% of CKB2$^{wt/wt}$ develop pancreatic tumors with an average latency of 366 and 406 d, respectively[41].

Skoulidis *et al*[42] described a mouse model PDX-1-Cre-Kras^{G12D} with two distinct mutant alleles of Brca2. The first encodes a germline truncating allele Brca2^{Tr} (Tr), that mimics Brca2 human mutations in pancreatic cancer, and the second is a conditional deletion (F11) in which LoxP sites flank Brca2 exon 11 and emulates the loss of heterozygosity observed in human cancers.

Homozygous Brca2 inactivation in KPCB2$^{Tr/\Delta 11}$ mice displays pancreatic cancer in high penetrance with rapid and predictable clinical decline. The median survival was 84 d compared with the KPCB cohort whose median survival was 168 d. Mice with germline heterozygosity for Brca2^{Tr} display pancreatic carcinogenesis, as even KCB$^{Tr/wt}$ mice with wild type Trp53 and mutant Kras-G12D in which pancreatic cancer is reported to develop less readily[39]. There is a reduction in PDAC-free survival of KCB$^{Tr/wt}$ mice in comparison with KCB controls with wild type Brca2. The pancreatic tumors observed in these mice display histological features similar to human pancreatic cancers with desmoplastic stroma. These tumors evolved with pancreatic intraepithelial neoplasia and metastatic behavior.

Interestingly, the KPCB$^{Tr/\Delta 11}$ mice which carry biallelic Brca2 mutations uniquely develop an acinar cell carcinoma component in 18% of cases, not observed in the other cohorts with Brca2 heterozygosity. This model shows that Brca2 inactivation promotes Kras-driven pancreatic malignancies[42].

Mist1$^{KrasG12D/+}$ transgenic model

To generate this transgenic model, Tuveson *et al*[25] used homologous recombination to target the expression of Kras^{G12D} to the Mist1 locus, a gene known to be expressed at earlier stages of pancreatic exocrine development. Mist1 is a basic helix-loop-helix transcription factor that is expressed at low levels in the embryonic pancreas at day 10.5[43,46,47] and in the adult, Mist protein is restricted to mature pancreatic acinar cell and is not found in ductal or islet cells[48,49]. Mist1$^{KrasG12D/+}$ mice have a diminished median survival of 10.8 mo compared with 24.2 mo in control wild type mice. Newborn mice show acinar hyperplasia with an increased proliferative index and acinar adenomas at 2 mo known as "acinar-ductal metaplasia". Metaplastic ductal structures with mucinous cytoplasm that resemble murine PanIN-IA are found in the pancreas in close association with metaplastic acini. These metaplastic ducts are

characterized by the presence of CK19 and acidic mucin staining with alcian blue. At three months of age they become cachectic with pancreatic tumors and metastasis. Most of these tumors are acinar although some of them are cystic papillary neoplasms with acinar differentiation. Surprisingly, these mice also develop early and advanced hepatocellular carcinoma and some of them succumb before invasive pancreatic carcinoma. Mist1$^{KrasG12D/+}$ mice die of advanced pancreatic exocrine carcinoma.

PDX1-Cre, Kras^{G12D}, Ink4a/Arf$^{flox/flox}$ transgenic model

As the loss of function of the G1 cyclin-dependent kinase inhibitor, INK4A, appears to be a near universal event in pancreatic adenocarcinoma when there is an alternate reading frame or distinct first exon in the INK4A/ARF locus[50-52], transgenic mice with this modification have been studied.

It was shown that mice with a constitutive deletion of both or either component of the Ink4a/Arf locus do not develop spontaneous pancreatic cancer[53]. Aguirre *et al*[23] demonstrated the cooperative interaction between Ink4 and Kras using mice engineered with Cre-mediated activation of mutant Kras (Kras^{G12D}) and the deletion of a conditional Ink4/Arf tumoral suppressor allele.

In this model, the LSL-Kras^{G12D} allele is expressed at the endogenous level after Cre mediates the expression of a transcriptional stopped element. The conditional Ink4a/Arf allele (Ink4/Arf^{flox}) was engineered to sustain Cre-mediated excision of exon 2 and 3, thereby eliminating p16^{Ink4} and p19^{Arf} proteins. The double engineered mouse expressed the Kras^{G12D} allele and lack of both copies of the conditional Ink4/Arf allele specifically in the pancreas after using the PDX-1-Cre transgene. Between 7 and 11 wk of age, PDX-1-Cre, Kras^{G12D} Ink4a/Arf$^{flox/flox}$ mice show weight loss, ascites, jaundice and pancreatic tumors ranging in diameter from 4 to 20 mm. These pancreatic tumors are highly invasive, frequently involving the duodenum, stomach and spleen but no liver or lung metastasis. Furthermore, invasion of the lymphatic and vascular system is detected, an observation suggestive of metastatic potential of these neoplasms.

Consistent with a ductal phenotype, the tumors are positive for CK-19, DBA lectin and show stromal collagen deposition. In contrast, they do not show reactivity for amylase and insulin.

In conclusion, KrasG12D expression in combination with Ink4a/Arf deficiency resulted in an earlier appearance of PanIN lesions and these neoplasms progressed rapidly to highly invasive and metastatic cancers, resulting in death in all cases by 11 wk.

PDX1-Cre, Kras^{G12D}, Smad4$^{flox/flox}$ transgenic model

Although selective SMAD4 has no discernable impact on pancreatic development or physiology, when combined with the activated KRAS^{G12D} allele, SMAD4 deficiency enabled rapid progression of Kras^{G12D}-initiated neoplasms including pancreatic tumors. The combination of Kras^{G12D} and SMAD4 deficiency resulted in the rapid development of tumors resembling intraductal papillary mucinous neoplasia (IPMN), a precursor to PDAC in humans. The SMAD4 tumor suppressor gene encodes a transcription factor that is a central effector of transforming growth factor-β (TGF-β)[30] and inactivating mutations in this gene are common in PDAC[54]. Bardeesy *et al*[55] generated a conditional knockout allele of Smad4 (Smad4^{lox}) harbouring loxP sites flanking exons 8 and 9 in the mouse germline. They crossed Smad4^{lox} homozygous mice to either the PDX1-Cre or Ptf1a-Cre transgenic mice. Mice with a homozygous deletion of Smad4 in the pancreas showed no evidence of any gross anatomic or physiological abnormalities, and exhibited normal pancreatic cytoarchitecture and differentiation.

In contrast, LSL-Kras^{G12D}-Smad4$^{lox/lox}$ mice showed low-grade PanINs and acinar-ductal metaplasia from 4 wk of age, an abdominal mass between 7 and 12 wk and reached terminal morbidity between 8 and 24 wk of age and a tumor-free survival of 13-15 wk. The pancreatic tumors were positive for cytokeratin 19, Shh, Hes1, phospho-stat3, mucin, Muc1, Muc4 and Muc5AC, but lacked acinar (amylase) and islet (insulin) marker expression. Mice showed palpable abdominal masses between 7 and 12 wk of age, and reached terminal morbidity between 8 and 24 wk of age.

Since the combination of Kras^{G12D} expression and Smad4 deletion showed a rapid onset of IPMN and advanced PanIN lesions, but exhibited only moderate pancreatic malignant progression, and since SMAD4 loss occurs with concurrent INK4A loss and Kras activation in human PDAC, the authors developed a transgenic mouse PDX1-Cre, Kras^{G12D} Ink4a/Arf$^{lox/lox}$ Smad4$^{lox/lox}$. These mice have significantly reduced survival, around 8 wk associated with PDAC and a small number of them also have IPMN and liver metastasis.

Ptf1a$^{cre/+}$, LSL-Kras$^{G12D/+}$, Tgfbr2$^{flox/flox}$ transgenic model

TGF-β signaling plays an important role in PDAC progression, as indicated by the fact that Smad4, which encodes a central signal mediator downstream from TGF-β, is deleted or mutated in 55% of human PDAC[54,56-58]. Pancreas-specific Tgfbr2 knockout mice have also been generated, alone or in the context of active Kras^{G12D} expression. Ijichi *et al*[27] crossed the LSL-Kras$^{G12D/+}$ mice with Tgfbr2 knockout mice[59] (previously developed) and generated mice of the genotype Ptf1a$^{cre/+}$, LSL-Kras$^{G12D/+}$, Tgfbr2$^{flox/flox}$. These mice had active KrasG12D expression plus Tgfbr2 knockout both in a pancreas epithelium-specific manner.

Ptf1a$^{cre/+}$, Tgfbr2$^{flox/flox}$ mice did not have pancreas development effects or discernable pancreatic cancer phenotype during 1.5 years.

In contrast, $^{Ptf1acre/+}$, LSL-Kras$^{G12D/+}$, Tgfbr2$^{flox/flox}$ mice had abdominal distension due to ascites, weight loss, and jaundice at 6-7 wk of age. Finally, these mice developed well-differentiated PDAC with 100% penetrance and a median survival of 59 d. Tumors are always accompanied by a whole panel of mPanINs and acinar-ductal metapla-

sia lesions from 3.5 wk and mice frequently have liver and lung metastases, direct invasion to the duodenum, and peritoneal dissemination.

While Ptf1a$^{cre/+}$, LSL-Kras$^{G12D/+}$, Tgfbr2$^{flox/+}$ mice show normal pancreas histology, tumors from Ptf1$^{acre/+}$, LSL-Kras$^{G12D/+}$, Tgfbr2$^{flox/flox}$ mice exhibited uniformly well-differentiated glandular architecture, which occupied the entire pancreas, resulting in almost complete loss of normal pancreatic tissue. Tumoral cells show positive ductal markers, CK19 and mucin, and are negative for the acinar and islet markers, amylase and insulin, indicating ductal adenocarcinoma. In addition, these tumors are rich in stromal component, positive for vimentin and smooth muscle actin staining.

In conclusion, Tgfbr2 knockout mice combined with Kras^{G12D} expression developed well-differentiated PDAC with 100% penetrance and a median survival of 59 d. Moreover, a distinct and important feature of this mouse model is that the Ptf1a$^{cre/+}$, LSL-Kras$^{G12D/+}$, Tgfbr2$^{flox/flox}$ tumors did not show sarcomatoid architecture, which was seen in one-third of the KrasG12D, Ink4a/Arf knockout model[23].

XENOGRAFT MOUSE MODELS

Tumor xenograft mouse models have been commonly used in preclinical studies for the last few years[60-62]. Human tumor xenograft models are created by the injection of human tumor cells grown from culture into a mouse or by the transplantation of a human tumor mass into a mouse. The xenograft may be readily accepted by immunocompromised mice such as athymic nude mice or severely compromised immunodeficient mice[63]. Xenografts show different advantages as they mimic genetic and epigenetic abnormalities that exist in tumors, can be used in the development of individualized molecular therapeutic approaches and can be implanted into the same organ to reproduce the organ microenvironment or the tumor[63].

There are two main types of human xenograft mouse models used for pancreatic cancer research, heterotopic and orthotopic, defined by the location of the implanted xenograft.

Heterotopic xenograft model

For heterotopic subcutaneous models, the xenograft is implanted between the dermis and underlying muscle and is typically located on the flank, on the back or the footpad of the mice. For many years, the subcutaneous xenograft model has been the most widely used preclinical mouse model for cancer research because it is rapid, inexpensive, reproducible, and has been considered sufficiently preclinical to test anti-cancer drugs. The subcutaneous model also has the advantages of providing visual confirmation that mice used in an experiment have tumors prior to therapy; and provides a means of assessing tumor response or growth over time, compared to intracavitary models where animal survival is the sole measure of response[64].

Different studies have used tumor engraftment in nude mice to study the possible response to chemotherapy treatment such as gemcitabine[65] or new pharmacological blocking agents[66] obtaining good results and suggesting new potential treatment options for pancreatic cancer.

One of the disadvantages of the heterotopic model is that it was observed that drug regimens that are curative in these models often do not have a significant effect on human disease as the subcutaneous microenvironment is not relevant to that of the organ site of primary or metastatic disease. Additionally, subcutaneous tumor models rarely form metastases. These observations suggest that heterotopic tumor models that do not represent appropriate sites for human tumors are not predictive when used to test responses to anti-cancer drugs[60,67,68].

Orthotopic xenograft model

Orthotopic tumors are transplanted to the appropriate organ in the mouse. For example, human pancreatic cancer cells are injected into the mouse pancreas and not into the skin on the mouse's back. Advantages of orthotopic models include use of the relevant site for tumor-host interactions, the development of metastases, the ability to study site-specific dependence of therapy, organ-specific expression of genes and the clinical scenario can be replicated. Major disadvantages are that orthotopic tumor xenograft generation is labor intensive, technically challenging, expensive, requires longer healing and recovery time and that monitoring tumor volume requires relatively lower throughput imaging methods[67]. Nonetheless, orthotopic tumor models are emerging as the preferred model for cancer research due to the increased clinical relevance.

To study pancreatic cancer, the standard procedure uses anesthetized mice 6-8 wk old. The abdominal skin and muscle are incised just off the midline and directly above the pancreas to allow visualization of the pancreatic lobes; the pancreas is gently retracted and positioned to allow direct injection of tumoral cells. The pancreas is replaced within the abdominal cavity; and both the muscle and skin layers are closed with surgical glue. Following recovery from surgery, mice are monitored and weighed daily to evaluate the tumor or response to treatment[61].

These models have been employed to study gene expression profiling of liver metastases and tumour invasion in pancreatic cancer[69] in basic research. In translational medicine, ortothopic models have been used to evaluate the antitumor efficacy of gemcitabine plus emodin[70].

In conclusion, different *in vivo* models of pancreatic cancer have been developed for the evaluation of multiple chemotherapeutic drugs and to study the molecular mechanisms implicated in resistance to different treatments.

These models are now available to investigate basic and translational aspects, but multiple considerations should be kept on mind for model selection depending on the purpose. The optimal model system should investigate

Table 2 Comparison of mouse models for the clinical approach in pancreatic cancer

Mouse model	Cost	Time consuming	Clinical approach	Clinical reproducibility (human disease)
Transgenic engineered	++++	++++	+	++++
Xenograft heterotopic	+	+	++++	+
Xenograft orthotopic	++	++	+++	++

+: Low; ++: Medium; +++: High; ++++: Very high.

invasiveness or metastasis, the criteria for assessing response and altered molecular pathways, expression of markers and time expression and tumor development are some of the most important factors (Table 2).

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REVIEW

Magnifying endoscopy in upper gastroenterology for assessing lesions before completing endoscopic removal

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Abstract

Any prognosis of gastrointestinal (GI) cancer is closely related to the stage of the disease at diagnosis. Endoscopic submucosal dissection (ESD) and *en bloc* endoscopic mucosal resection (EMR) have been performed as curative treatments for many early-stage GI lesions in recent years. The technologies have been widely accepted in many Asian countries because they are minimally invasive and supply thorough histopathologic evaluation of the specimens. However, before engaging in endoscopic therapy, an accurate diagnosis is a precondition to effecting the complete cure of the underlying malignancy or carcinoma *in situ*. For the past few years, many new types of endoscopic techniques, including magnifying endoscopy with narrow-band imaging (ME-NBI), have emerged in many countries because these methods provide a strong indication of early lesions and are very useful in determining treatment options before ESD or EMR. However, to date, there is no comparable classification equivalent to "Kudo's Pit Pattern Classification in the colon", for the upper GI, there is still no clear internationally accepted classification system of magnifying endoscopy. Therefore, in order to help unify some viewpoints, here we will review the defining optical imaging characteristics and the current representative classifications of microvascular and microsurface patterns in the upper GI tract under ME-NBI, describe the accurate relationship between them and the pathological diagnosis, and their clinical applications prior to ESD or *en bloc* EMR. We will also discuss assessing the differentiation and depth of invasion, defying the lateral spread of involvement and targeting biopsy in real time.

Key words: Magnifying endoscopy with narrow-band imaging; Upper gastroenterology; Assessment; Endoscopic submucosal dissection; Endoscopic mucosal resection

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INTRODUCTION

Gastrointestinal (GI) cancer is a major medical and economic burden worldwide. Esophageal and gastric cancers remain a considerable source of morbidity and mortality in Asian countries. For instance, in Linxian, Henan province (China), cancer of the upper GI tract is endemic.

Mortality rates for esophageal cancer in Linxian exceed the American average (for white men) one hundredfold[1]. The prognosis of GI cancer is closely related to the stage of disease at diagnosis, and most cases are still detected at advanced stages and result in a relevantly poor outcome[2]. Early detection of these neoplasms or their precursors may be the only chance to reduce this high mortality.

Early GI cancers - such as Barrett's esophagus (BE) with high-grade dysplasia and early gastric cancer (EGC)-whose invasion is limited to the mucosa or submucosa regardless of the size or the presence of regional lymph-node and distant metastasis[3], confer a survival rate of greater than 90% in 5 years in many centres[4,5].

The screening program for gastric cancer in Japan indicates that 53% of diagnosed gastric cancers are localized lesions. Additionally, the accumulated clinical experience and formal outcome studies have shown that the majority of early-stage neoplastic lesions is localized with a low risk of lymph node metastasis. Recent data from 3261 patients who underwent gastrectomy with meticulous D2-level lymph node dissection over a 30-year period show that lymph node invasion was observed in only 2.7% of mucosal tumors and 18.6% of EGC invading the submucosa[6]. Clinical experience suggests that complete resection of the cancer is possible, and a cure can be achieved as long as the potential for metastatic spread is definitively excluded[7,8].

Based on the above knowledge, the doctors began to try to use endoscopes for local excision with GI early tumors *in situ*, invading lamina propia or submucosa. More than a decade ago, endoscopic mucosal resection (EMR) technique emerged first in Japan as a critical tool in the management of patients with both high-grade dysplasia and superficial carcinomas[9]. But the indication of EMR is generally limited to mucosal tumors less than 2 cm in size even with the series of improvements that have been most widely used in recent years, such as using a transparent cap-fitted endoscope to suck targeted lesions into the cap and resect them with a snare (EMR-C) or a ligation device (EMR-L). All above EMR technologies are difficult to resect *en bloc* tumors larger than 2 cm in size, which is required for accurate and reliable pathological examination. However, though some endoscopists adopt piecemeal EMR techniques in order to cure the larger lesions, further investigation has revealed it involves problems such as remnants or high recurrent rates due to incomplete resections[10]. Thus, to overcome the problem of EMR techniques, a recent key issue in the field of therapeutic endoscopy is the development of a new therapeutic strategy for early GI cancers using endoscopic submucosal dissection (ESD). In this procedure, submucosal dissection is carried out by using an electrocautery knife to acquire a single-piece specimen, which is the gold-standard technique for offering *en bloc* resection of large superficial tumors in the GI tract, especially when R0 resection cannot be performed with other resection techniques. Within only a few years, ESD has become widespread in Asian countries - such as Japan, Korea and China - where there is a large volume of early upper GI lesions that need endoscopic treatment. However, there are hardly any reports about long-term results after ESD, and the procedure involves a much higher complication rate and requires much higher skills[11,12].

The two endoscopic local procedures are increasingly accepted by many patients and doctors mainly because they (1) provide new alternatives for minimal invasiveness; (2) are perhaps the first approximations to true intraluminal resection of superficial malignant GI neoplasms; and (3) yield results that are comparable to surgery. They also result in lower morbidity rates, lower costs and better quality of life than traditional surgery because of tissue preservation. But the difficulty lies in achieving *en bloc* or R0 resection and getting improved survival that precisely assesses resection margins and the depth of malignant invasion prior to performing EMR or ESD. The lesions with undifferentiated histology, lymphatic or vascular involvement and submucosal invasion were excluded due to possible lymph node metastases[3].

Therefore, a thorough preoperative endoscopic examination is considered necessary for selecting the appropriate therapeutic modality. Due to this requirement, endoscopic equipment has improved markedly with respect to resolution in recent years. However, in 1967, Okuyama *et al*[13] produced a magnification endoscope for viewing the gastric mucosa. At present, magnification endoscopes have the ability to enlarge the image from 1.5 × to 150 × and produce images that have pixel densities as high as 850 000, allowing the discrimination of objects that are only 10-71 μm in diameter[14]. The newest magnification endoscopes permit magnification without loss of resolution[15]. Nevertheless, it was reported recently that some GI disorders, such as intestinal metaplasia, often appear translucent when observed with magnification endoscopy alone. Thus, the mucosal surface cannot be easily examined without staining[16]. Methylene blue, Lugol's iodine, and indigo carmine are several topical stains or pigments that have been used in conjunction with magnification endoscopy to improve tissue localization, characterization, or diagnosis during endoscopy[17]. The technique known as magnification chromoendoscopy (MCE) has been applied in a variety of clinical settings and throughout the GI tract for more than 10 years. In addition, other newer technologies, including narrow band imaging (NBI), that have proved particularly helpful during gastrointestinal endoscopic examinations have been developing in recent years. This shows that the two techniques have a similarly high sensitivity for detecting early neoplasia in the upper GI tract[18,19]. However, compared with MCE, the "electronic dyeing endoscopy," such as NBI, that are based upon the phenomenon that the depth of light penetration depends on its wavelength, are more user-friendly because their filters can be manually enabled and disabled during endoscopy, making it easy to switch them between the standard mode and the "electronic dyeing" mode, and no staining agents are required. Beyond these practical advantages, NBI reveals the superficial capillary network with a high contrast due to absorption of the blue light by hemoglobin, whereas the vascular pattern is

often less visible in chromoendoscopy[20]. When magnifying endoscopy is combined with narrow band imaging (ME-NBI), the combination has been shown to enhance visualization of the micromucosal and microcirculatory structure for a more detailed assessment of the early lesions[21].

Hence, in many institutions, especially in Japan, MCE or the ME-NBI technique has been extensively included in standardized procedure and is performed in addition to conventional white-light endoscopy prior to ESD or EMR[22]. For the colorectum, "Kudo's Pit Pattern Classification" has begun to be widely adopted by many endoscopists because it appeared valuable in the histological prediction from the observation of five-types pit patterns by MCE or NBI-although the microvascular observation is helpful as well[23]. In the upper GI, despite numerous studies from investigators around the world and especially in some Asian countries, there is still no consistent classification diagnosis system for ME-NBI before the endoscopic removal of esophageal and gastric lesions; each medical institution tends to adopt its own classification[24-36]. Therefore, here we will comprehensively review the literature in recent years on the main characteristics of microsurface (MS) and microvascular patterns, introduce their classifications that have become relatively popular in some Asian countries under ME-NBI, describe the accurate relationship between them, the pathological diagnosis for early lesions in the upper GI tract, and their clinical utility in ESD or *en bloc* EMR. We do this to help build consensus on observation flowcharts of ME-NBI and to help endoscopists recognize tthe classification of early upper GI lesions more clearly so that they can select the most appropriate therapeutic intervention.

DEFINING OPTICAL IMAGING CHARACTERISTICS VISUALIZED UNDER MAGNIFYING ENDOSCOPY WITH NARROW BAND IMAGING IN UPPER GASTROINTESTINAL

In general, the doctor inspects the patient first under white-light endoscopy without magnification. He then slowly moves the scope, washes the tissue well, and pays special attention to areas containing slight differences. The key endoscopic finding by using white light (WL) has been reported to be a change of color (slight redness) and pallid mucosa[37]. However, the margin is difficult to identify by conventional WL. Then, the NBI model was employed to make it easier to detect the change in colors and structure of the mucosa. Moreover, with magnification, the microvascular (MV) pattern and MS pattern can be evaluated. So, what will be seen under ME-NBI if the cancerous lesion is suspected within the area?

Esophagus

Brownish area: A brownish area can often be recognized by NBI observation as distinct boundaries are formed between the tumor lesion and normal epithelium (Figure 1)[38]. An intraepithelial papillary capillary loop (IPCL) appears as brown dots under NBI-enhanced observation. For example, in the esophagus, if the lesion appears brownish under magnifying NBI observation, it will predict the possibility of mucosal squamous-cell carcinoma as a result of assessing the morphologic changes in the IPCL. The brownish areas in the esophagus visualized by NBI generally correspond to the Lugol chromoendoscopy displayed the lesions as unstained areas[39].

Intraepithelial papillary capillary loop: It is well known that angiogenesis plays a critical role in the transition from premalignant to malignant lesions. Consequently, early detection and diagnosis based on morphological changes to the microvessels are crucial[40]. Superficial blood vessels in the esophageal mucosa consist of branching vessels and IPCL. However, in some cases, only the former can be observed under the WL that extend to the horizontal plane and exist immediately above the muscularis mucosa while IPCL that rises perpendicularly from a branching vessel can be observed through ME-NBI (Figure 2)[41]. In these cases, Muto *et al*[43] have reported that a well-demarkated brownish area or an area of scattered brownish dots under NBI is connected with the proliferation of IPCL. This is a useful indicator for early esophageal squamous-cell carcinoma or high-grade intraepithelial neoplasia.

Stomach

Besides the MV architecture, the imaging characteristics of the MS structure of mucosa the so-called pit or crypt patterns can be obtained by ME-NBI in the stomach (Figure 3).

Subepithelial capillary network and collecting venule: By ME-NBI, the subepithelial capillary network (SECN) and the collecting venule (CV) can be clearly visualized. A polygonal-shaped subepithelial capillary loop surrounding each pit forms a network in a regular arrangement, and this capillary network drains into a CV. SECN and CV are basic anatomical components for analysis of the MV architecture. The SECN shows two distinct patterns depending on the region of the normal stomach being imaged: The body mucosa demonstrates a regular honeycomb-like SECN pattern with a CV, whereas the gastric antrum shows a coil-shaped SECN but the CVs are rarely observed. This might be because the CVs in the antral mucosa are relatively deeper from the surface epithelium than those of the gastric body mucosa[44].

For the abnormal stomach, there are two characteristics of MV that can be identified by ME-NBI: the first is a relatively regular "fine network pattern" (Figure 3A), which is more likely to be observed in well-differentiated adenocarcinoma and appears as mesh and abundant microvessels connected with each other; the second is a "corkscrew pattern" (Figure 3B) as with isolated and tortuous microvessels, which often represents the low density of MV and corresponds to poorly-differentiated,

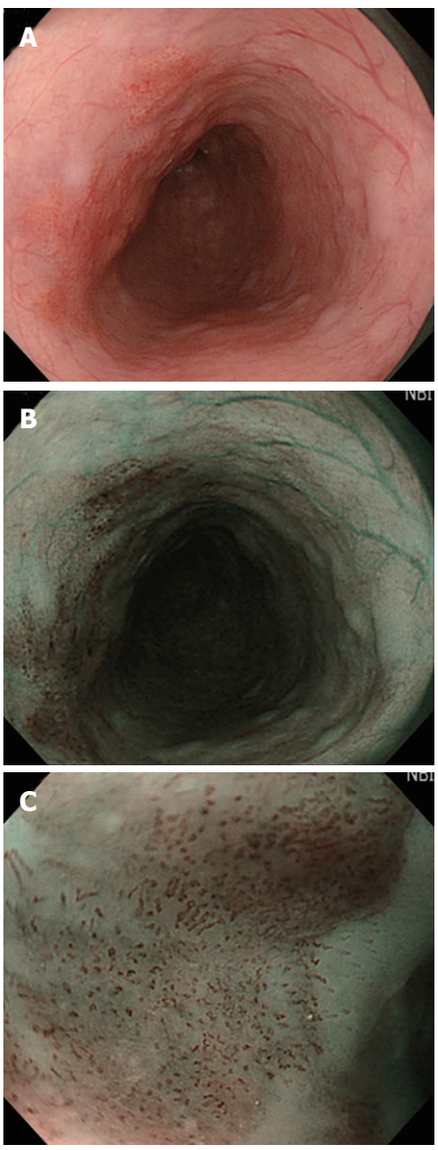

Figure 1 The carcinoma visualized in esophagus. A: Carcinoma in esophagus is difficult to identify by conventional white light; B: Carcinoma in esophagus can be easily recognized by narrow-band imaging (NBI) as well-demarcated brownish area; C: Intraepithelial papillary capillary loop can be observed by magnifying endoscopy with NBI at the edge of the tumor.

depressed (0-Ⅱc), early gastric adenocarcinoma[45].

Intrastructural irregular vessel (ISIV) (Figure 3C) also has an irregular MV pattern but often appears in the superficial flat gastric lesion (0-Ⅱb) as well as the marginal flat area of an elevated or a depressed lesion. This is a cancerous indication. Differing from the fine network pattern and corkscrew pattern shown in the areas where fine mucosal structure (FMS) disappear or are unclear in 0-Ⅱc gastric lesions, the ISIVs are found enclosed in villous or papillary FMSs and have characteristics of dilation, heterogeneity, abrupt caliber or tortuousness of shape[46].

Microsurface: Applying ME-NBI is helpful for clearly visualizing not only some of the MV characteristics introduced above but also the gastric mucosal MS structures,

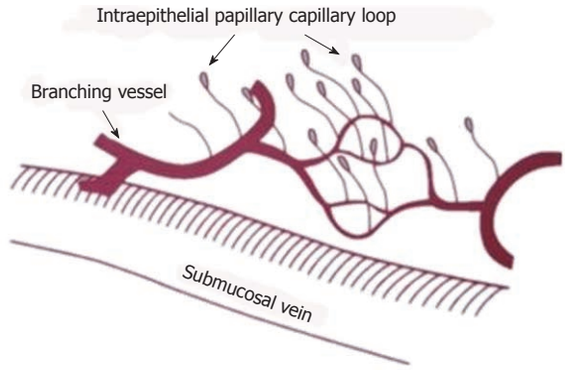

Figure 2 The superficial blood vessels in the squamous esophagus (from Inoue *et al*[42], with permission, making a little change for the original graph), the intraepithelial papillary capillary loop rises from the branching vessel and terminates in a diffuse.

namely pit or crypt opening patterns. The MS structures include the FMS in a normal stomach as well as the irregular or loss of pit pattern that occurs with early gastric carcinomatous lesions.

Although it is necessary to assess a neoplasm in the stomach by the MV and MS patterns simultaneously, it is sometimes impossible to visualize the subepithelial MV pattern on account of overcurtaining by the white opaque substance (WOS). In most adenomatous lesions, the WOS is frequently observed more clearly under ME-NBI than WL and is speculated to be some intracellular component within the neoplastic epithelium of the intervening part between the crypts, obscuring the morphology of the subepithelial MV and causing difficulty in assessing the MV pattern. In such cases, rather than assessing the MV pattern, the morphology of the WOS could be an alternative new optical microstructure sign for distinguishing adenomas from adenocarcinomas. Yao *et al*[47] reported that only about 6% of the WOS was found in Ⅱb and Ⅱc lesions. For 0-Ⅱa type neoplasms, the WOS was more frequently visualized in adenomas (78%) than in carcinomas (43%) and showed a well-organized and symmetrical distribution of the dense WOS of a regular reticular/maze-like/speckled pattern (Regular WOS) (Figure 3D) within adenomas (100%), but showed a disorganized and asymmetrical distribution of the fine WOS of irregular reticular/speckled pattern (Irregular WOS) (Figure 3E) within carcinomas (83%). That is to say, the regular WOS is characteristic of adenomas, whereas its irregular distribution is characteristic for carcinomas.

Similar to the WOS, the light blue crest (LBC) (Figure 3F) is another characteristic optical microstructure under ME-NBI caused by the dense reflection of 400 to 430 nm short-wavelength light at the ciliated tissue. The LBC is defined as a fine, blue-white line on the crests of the epithelial surface/gyri, just at the edge of crypts. It has been suggested that the appearance of the LBC on the epithelial surface of the gastric mucosa may be a distinctive endoscopic finding associated with the presence of histological intestinal metaplasia in high sensitivity (89%), high specificity (93%), and high accuracy (91%)[48]. The

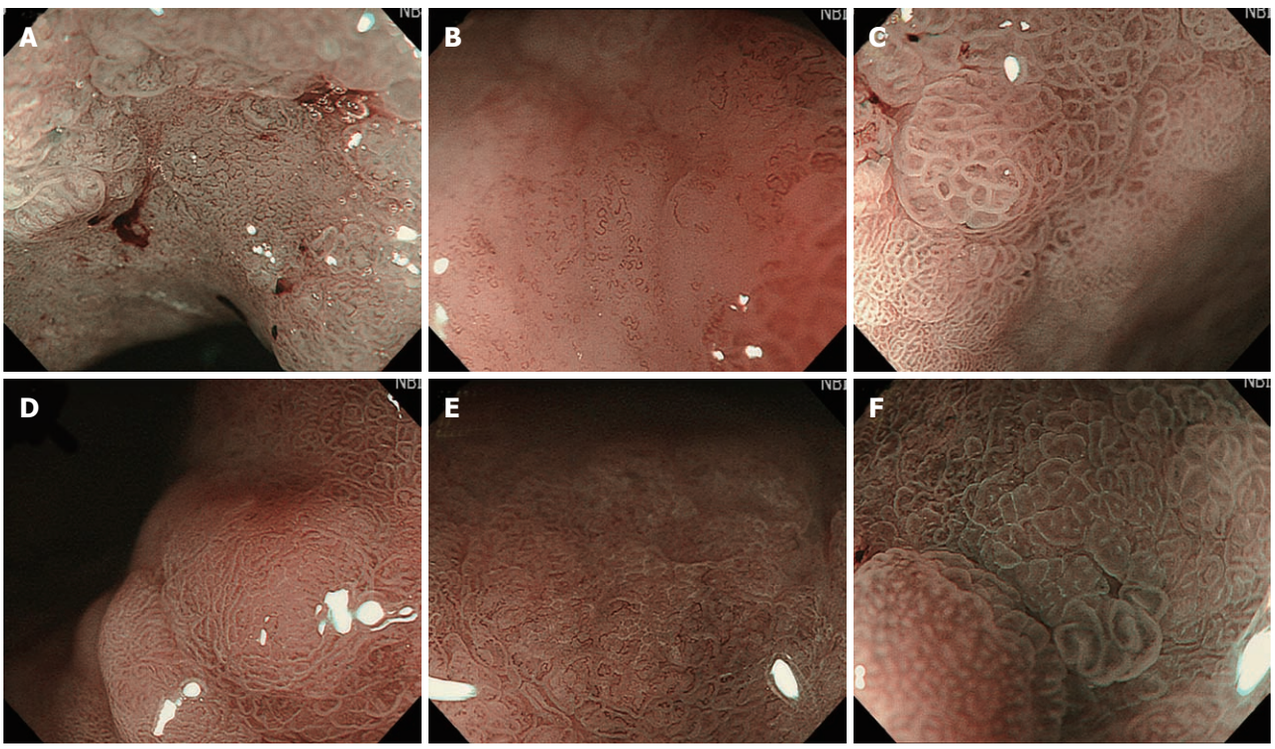

Figure 3 Some typical microvascular and microsurface imaging characteristics visualized in stomach under magnifying endoscopy with narrow band imaging. A: Fine network pattern, mostly corresponding to well-differentiated adenocarcinoma (0-Ⅱc, gastric); **B: Corkscrew pattern, mostly corresponding to the** poorly-differentiated adenocarcinoma (0-Ⅱc, gastric); **C: Intrastructural irregular vessel, enclosed in villous or papillary fine mucosal structure, had irregular shape** characters such as dilation, heterogeneity, abrupt caliber or tortuousness (0-Ⅱb, gastric); **D: Regular white opaque substance (WOS), that shows well-organized and** symmetrical distribution with a regular reticular pattern and obscures the subepithelial microvascular (MV) pattern (0-Ⅱa adenoma, gastric); **E: Irregular WOS, that is** present within the cancerous epithelium with an irregular speckled pattern and makes the subepithelial MV pattern cannot be clearly visualized (0-Ⅱa cancer, gastric); **F:** Light blue crest, defined as a fine, blue-white line on the crests of the epithelial surface in the gastric mucosa may be a distinctive endoscopic finding associated with the presence of histological intestinal metaplasia.

LBC was also demonstrated to have a significant association with gastric atrophy and a high occurrence of gastric cancer[49].

As noted above, the strategies for diagnosing upper GI lesions by ME-NBI are specific to different organs. Under a magnifying endoscope, an esophageal neoplasia could be diagnosed solely according to the findings from the MV pattern, namely IPCL, because the esophageal squamous epithelium does not show FMS. In contrast, a gastric neoplasia could be diagnosed with the findings of the MV pattern as well as the MS pattern[50,51]. Of course, sometimes the WOS or LBC is more useful for the diagnosis.

CURRENT REPRESENTATIVE CLASSIFICATIONS OF MICROVASCULAR AND MICROSURFACE PATTERNS IN THE UPPER GASTROINTESTINAL UNDER MAGNIFYING ENDOSCOPY WITH NARROW BAND IMAGING

Classifications of intraepithelial papillary capillary loops in the esophagus

IPCLs beneath the basement membrane of the esophageal squamous epithelium can be observed by ME-NBI. It has been shown that identifying IPCL changes is very important in predicating early lesions of the esophagus. Regarding the classifications of IPCLs, there have been several systems adopted by different researchers[26-28], but in Japan, Inoue's classification and Arima's classification of IPCLs have been relatively popular.

Inoue's classification of intraepithelial papillary capillary loop: Under NBI, the **IPCLs are easily recognized** as brown spots, and the normal patterns appear as a smooth-running, small-diameter capillary vessel in the normal epithelium. The abnormal shapes appear as four typical changes: **Dilation, tortuous weaving, irregular caliber** and form variation. Inoue *et al*[24,52] classed them into five types and several subtypes from type I to type V-N as below (Table 1 and Figure 4). IPCLs in type I is no different from the normal pattern. IPCLs in type Ⅱ has one or two different characteristics: elongation and/or dilation is often seen. IPCLs in type Ⅲ have no or few differences from the normal pattern, but this type differs from type I mainly in the features of color changes under NBI and iodine staining. Under NBI, the lesions of type I and type Ⅱ often show no change or negligible change, but the types between type Ⅲ and type V-N appear brownish. **In addition, type** I and type Ⅱ lesions are

Table 1 Inoue's classification of intra-papillary capillary loop in esophagus

Typing	IPCL	Iodine staining	Under NBI	Pathological assessing	Treatment
Type Ⅰ	Smooth running small diameter capillary vessel with no difference from the normal pattern	Stained		Normal epithelium	
Type Ⅱ	Elongation and/or dilation capillary is often seen	Slightly stained		Esophagitis or re-generative tissue	
Type Ⅲ	No or minimal change from the normal	Unstained	Brownish	HGIEN	Further follow-up
Type Ⅳ	Showing two or three of four patterns among dilation, meandering, caliber changes and different shapes	Unstained	Brownish	HGIEN or m1 carcinoma *in situ*	ESD/*en bloc* EMR
Type Ⅴ	Demonstrating all four characteristic changes: dilation, tortuous weaving, irregular caliber and form variation	Unstained	Brownish	M1 carcinoma *in situ*	
Type Ⅵ	Elongation basing on the shapes of type Ⅴ IPCL , keeping IPCL partly	Unstained	Brownish	M2 carcinoma *in situ*	
Type Ⅶ	Destructing dramatically and running on horizontal plane	Unstained	Brownish	M3-Sm1deeper carcinoma	Relatively indicated for ESD/EMR
Type Ⅷ	New tumor vessel appear	Unstained	Brownish	Sm2 deep carcinoma	Surgery, chemorado-therapy

IPCL: Intraepithelial papillary capillary loop; NBI: Narrow-band imaging; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; HGIEN: High-grade intraepithelial neoplasia.

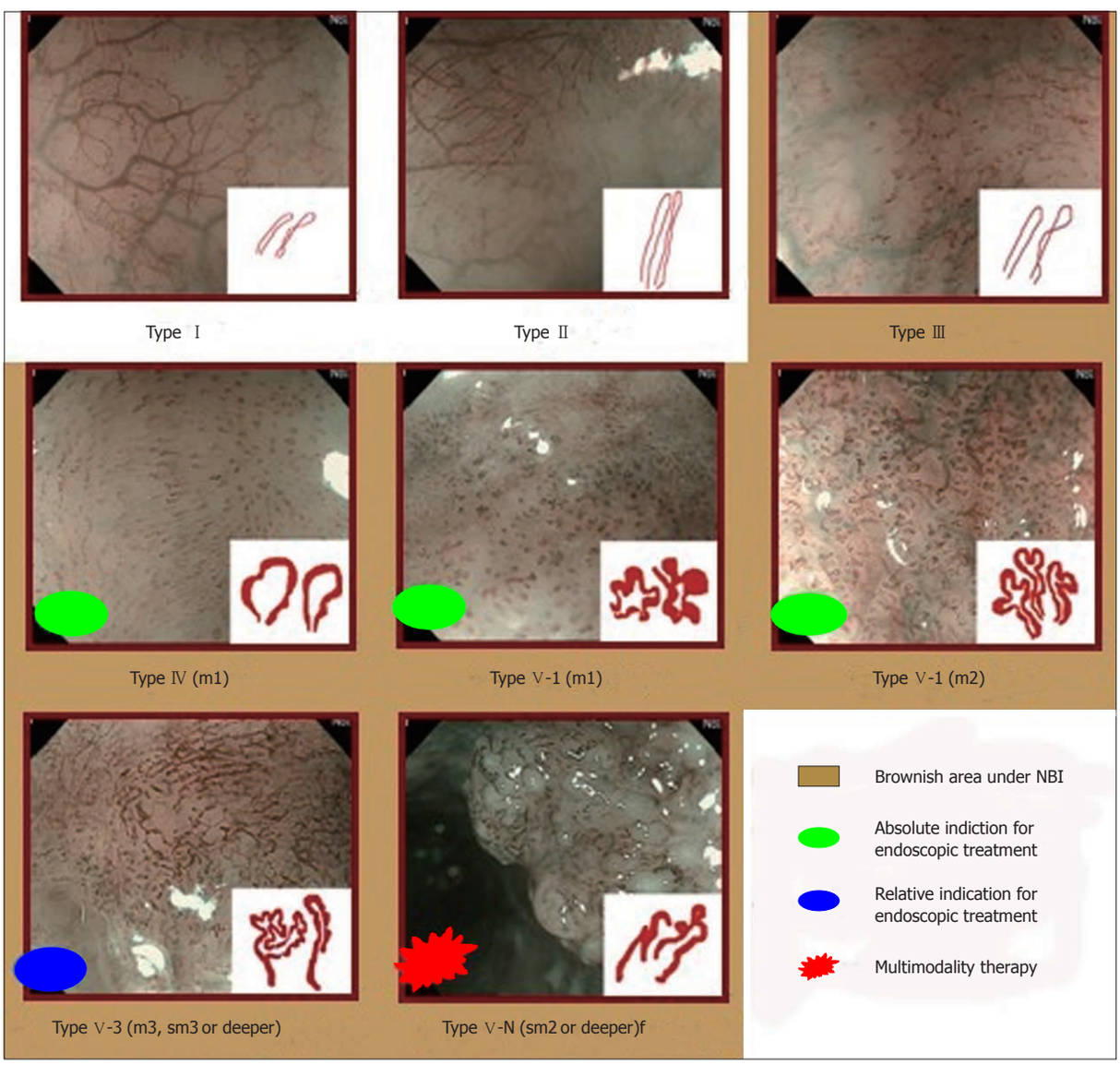

Figure 4 The case examples of Inoue's intraepithelial papillary capillary loop classification from type Ⅰ to type Ⅴ-N. NBI: Narrow-band imaging.

often positively stained with iodine while the types from type Ⅲ to type Ⅴ-N are negatively stained. IPCLs in type Ⅳ appear to have two or three of the four abnormal characteristic changes. IPCLs in type Ⅴ-1 demonstrate all the four typical changes. IPCLs in type Ⅴ-2 are elongated on the base of the four shapes and only keeping part of the original IPCL. IPCLs in type Ⅴ-3 are further degraded and run on a horizontal plane. As for type Ⅴ-N, the most remarkable feature is the appearance of new tumor vessels.

According to the grade of the changes of IPCL, the depth of invasion can be assessed. Type Ⅰ mainly appears in normal epithelium. Type Ⅱ corresponds to inflammatory changes or regenerative tissue. Type Ⅲ often reflects low-grade intraepithelial neoplasia. Type Ⅳ is linked to with high-grade intraepithelial neoplasia (HIN) or M1 carcinoma *in situ*. Type Ⅴ-1 is definitively diagnosed as M1 carcinoma *in situ*. The appearance of Type Ⅴ-2 strongly suggests m2 carcinoma. Type Ⅴ-3 often indicates m3 to sm1 deep lesions. Type Ⅴ-N is often associated with sm2 invasion cancer. In short, type Ⅰ to type Ⅴ-1 demonstrate the characterization for flat lesions while type Ⅴ-1 to type Ⅴ-N reflect invasive cancers.

With treatment, lesions of type Ⅲ IPCLs need further follow-up, and type Ⅳ to type Ⅴ-2 should be considered for ESD or *en bloc* ESD. Type Ⅴ-3 lesions are thought to be an indication for ESD or EMR because of the depth of invasion ranges between m3 and sm1. A complete biopsy should be applied before deciding on a treatment strategy. For type Ⅴ-N, it is taken for granted that the surgical treatment or chemoradiotherapy should be recommended to counteract the significantly increasing risk of lymph node metastasis.

Arima's classification of intraepithelial papillary capillary loop: In 2005, Arima *et al*[25] reported another classification of the microvasculature of esophageal IPCLs under magnifying endoscopy. The microvascular patterns are categorized into four types (Figure 5). The thin, liner capillaries in subepithelial papillae are recognized as type Ⅰ, resembling the shapes in normal mucosa. The vessels of type Ⅱ become distended and dilated in subepithelial papillae, and the structure of capillaries is preserved. Most of them are usually found in lesions with inflammatory changes and are also associated with intraepithelial neoplasia. Spiral vessels with an irregular caliber and crushed vessels with red spots are characteristics of type Ⅲ, which are often seen in m1 or m2 cancers. Type Ⅳ usually appears to be irregularly multilayered, irregularly branched, reticular vessels with an irregular caliber as generally observed in cancers with an m3 invasion or deeper. Avascular areas as well as stretched vessels are seen in cancers with downward growth. In addition, reticular vessels are commonly seen in poorly differentiated cancers, and the size of a vascular area surrounded by distended vessels is related to the depth of tumor invasion.

Comparing to the above two classification systems on the morphologic changes of IPCL and predicting the depth of the tumor invasion, it can be argued that type Ⅰ of Arima's classification partly corresponds to type Ⅰ -type Ⅲ of Inoue's classification. Furthermore, type Ⅱ of Arima's classification partly corresponds to Inoue's type Ⅳ, Arima's type Ⅲ partly to Inoue's type Ⅴ-1 or Ⅴ-2, and Arima's type Ⅳ partly to Inoue's type Ⅴ-3 or Ⅴ-N. However, the two systems do not always have such clear corresponding links. The invasion depth diagnosis by Inoue's classification is possible for most lesions, and the correct ratio is about 78%[24,52]. By contrast, when using Arima's type Ⅲ and type Ⅳ classifications as diagnostic criteria for HIN and cancers, the rate of differential diagnosis goes up to 99%[25]. Recently, it has been reported[53] that some flat areas are not able to be predicted by Inoue's classification. However, combining the two classification systems could result in greater accuracy of the preoperative diagnosis, which is proved by the pathological diagnosis after ESD. Therefore, it is recommended for clinical endoscopists using Inoue's classification and Arima's classification together to make an invasion depth diagnosis of esophageal cancer under ME-NBI.

Stomach

As for the MS of the stomach, in 1978, Sakaki *et al*[54] described the gastric pit appearances under magnifying endoscopy and classified them into five types: (1) foveolar pattern; (2) foveo-intermediate pattern (FIP); (3) foveolo-sulciform pattern; (4) sulciform pattern; and (5) mesh pattern. Although "Sakaki's classification" is still currently the most widely adopted classification by many Japanese endoscopists, not all gastric pathological changes can be expressed by this system because it is not consistent with structural changes under some pathological conditions[55], which were found to have round and long elliptical gastric pits. The width of the FIP band seems to be related to the severity of atrophic gastritis, and the FIP is considered to indicate the position of the atrophic border.

Therefore, in 2002, Yagi *et al*[56] first reported a new modified classification system named the "A-B classification system," which is useful to describe typical micromucosal structures related to the development of *Helicobacter pylori* (*H. pylori*) gastritis. They classified the morphological changes in the glandular structure and microvascular architecture obtained by WL magnifying endoscopy into four types: (1)type Z-0: Gastric round pits resembling pinholes surrounded by a regular arrangement of collecting venules with SECN forming a network; (2) type Z-1: Irregular true capillaries but no collecting venules observed; (3) type Z-2: White gastric pits and sulci with neither collecting venules nor true capillaries being seen; and (4) type Z-3: Dilated pits with surrounding redness. Type Z-0 specifically indicated the *H. pylori*-negative mucosa and differed significantly from types Z-1, Z-2 and Z-3 with regard to the grade of inflammation, activity and presence of *H. pylori*.

More recently, with the development of brand new optical techniques, such as ME-NBI, which can clearly visualize not only the glandular structure but also the mucosal microvascular architecture in units as small as the

capillary, the prior diagnostic classification system seemed less able to meet clinical needs, especially for early diagnosing of premalignant lesions and assessing the relationship between microvessel patterns, pit patterns and histological patterns ahead of endoscopic *en bloc* resection. In recent years, many researchers modified the above classifications but varied individually[45,57-61], and there is still no set of consistent classification guidelines. Nonetheless, the key characteristic findings of all the current classifications for ME-NBI with respect to early gastric carcinomatous lesions are based on the types of abnormal MV patterns and irregular MS patterns. Among these, the representative diagnostic system is advocated by Yagi *et al*[62], who established a flowchart for ME-NBI diagnosis in early gastric cancerous lesions as below: first, the "white zone" should be imaged, which is Yagi's term for the border of the uniform or heterogeneous papillae in the mucosal MS structure that appears as a bold white line. Next, microvessels should be observed. A regular MV pattern means the microvessels appear regular in shape and arrangement and look like closed or open loops of uniform size caliber. An irregular MV pattern means the microvessels appear irregular in shape and arrangement, looking like tortuous or irregular branches of various sizes or abnormal caliber[47]. Then, according to the white zone, the MV pattern, the WOS, and the LBC, the histological imaging of entire mucosa should be done. (1) Fine network patterns and loop patterns are mostly associated with well- or moderately-differentiated adenocarinoma; (2) irregular MV patterns, namely ISIVs, enclosed in villous or papillary FMSs can often be observed in Ⅱb gastric cancerous lesions; (3) corkscrew patterns or wavy microvessels mostly correspond to the poorly-differentiated adenocarcinoma; (4) regular WOSs often appear in Ⅱa gastric adenoma lesions while irregular WOSs often present in Ⅱa gastric cancerous lesions; and (5) LBC is mostly connected with intestinal metaplasia[47,48,63].

As a matter of course, with regard to the classification of early gastric lesions under ME-NBI, more in-depth studies are needed to address the more morphologically-complex microstructures of the stomach relative to the other parts of the GI system. Some features described previously are not general enough to apply to each lesion, and the number of cases in the studies is limited as well. At present, it is reasonable to use ME-NBI as a supplementary diagnostic tool to normal endoscopy with chromoend-oscopy in the stomach before deciding on therapy strategies. The current strategies require new additions and some modifications.

Barrett's esophagus

BE is thought to be a complication of longstanding gastroesophageal reflux and a condition of the distal esophagus where normal squamous lining is replaced by columnar epithelium containing specialized intestinal metaplasia (SIM), which has the tremendous potential for developing esophageal adenocarcinoma with generally poor prognoses and a median survival rate of less than one year. Short BE is defined as < 3 cm and long BE as ⩾ 3 cm[64].

Using ME-NBI allows clear visualization of micromucosal and vascular patterns in BE. Now, depending on which targeted biopsy technique can be performed, improved distinction of nondysplastic SIM from HIN is possible. Recently, several pieces of literature[16,33,65,66] have reported their own classification systems, of which the principal features are summarized as follows: SIM is characterized by the mixing of villous, tubular and linear patterns with mostly regular arrangements and having regular vascular patterns or appearing as long, branching vessels in a flat mucosa. In addition, absent microstructural patterns also have a very high correlation to and predictive power for SIM. HIN is characterized by irregular/disrupted microstructural and irregular microvascular patterns, and the frequency of abnormalities shows a significant rise with increasing grades of dysplasia.

USEFULNESS OF MAGNIFYING ENDOSCOPY WITH NARROW BAND IMAGING PRIOR TO ENDOSCOPIC SUBMUCOSAL DISSECTION OR *EN BLOC* ENDOSCOPIC MUCOSAL RESECTION

To assess the differentiation and depth of invasion

Criteria for endoscopic submucosal dissection/endoscopic mucosal resection: Only some differentiation and invasion limited to sm1 lesions should be considered for endoscopic removal. Nowadays, in Asian countries, one of the widely adopted guidelines for ESD or *en bloc* EMR is that the histology of the tissues must be intramucosal, well-differentiated, early carcinoma, and the minute invasion of submucosal lesions must be limited to sm1-namely, with a depth less than 200 μm in the squamous epithelium of the esophagus and less than 500 μm in the stomach. If the lesion is recognized as undifferentiated, surgery should be recommended[6,67].

Japan's data show that the five-year cancer-specific survival rates of EGC limited to the mucosa and submucosa are 99% and 96%, respectively[67]. In other words, *en bloc* endoscopic treatment should be mainly applied to some category 0 superficial GI neoplastic lesions with the invasion limited to the mucosa or submucosa. These are divided into three subtypes according to the "Paris classification": 0-Ⅰ include Ⅰp and Ⅰs, referring to polypoid pedunculated and sessile respectively; 0-Ⅱ are non-polypoid and non-exca vated, and they are further subdivided into 0-Ⅱa for slightly elevated lesions, 0-Ⅱb for completely flat lesions, and 0-Ⅱc for slightly depressed lesions; 0-Ⅲ are non-polypoid with an ulcer (Figure 6, left side)[68]. In order to get a more precise evaluation for choosing the appropriate therapy, endoscopists classify early GI cancer into the following subdivisions according to the depth of invasion: M1, carcinoma with questionable invasion carcinoma limited to the epithelium; m2, cancer invasion to the lamina propria; m3, cancer infiltration into the muscularis mucosa; sm1, to the upper third

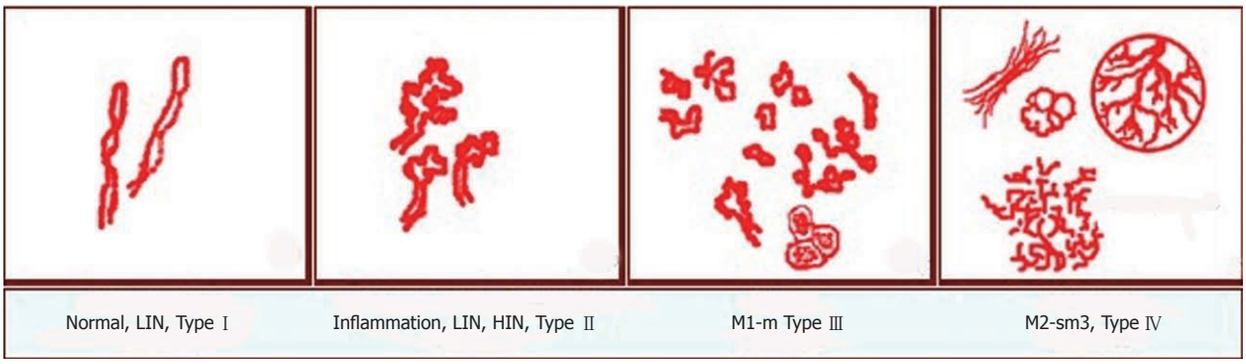

Figure 5 The morphology of Arima's intraepithelial papillary capillary loop classification in esophagus. LIN: Low-grade intraepithelial neoplasia; HIN: High-grade intraepithelial neoplasia.

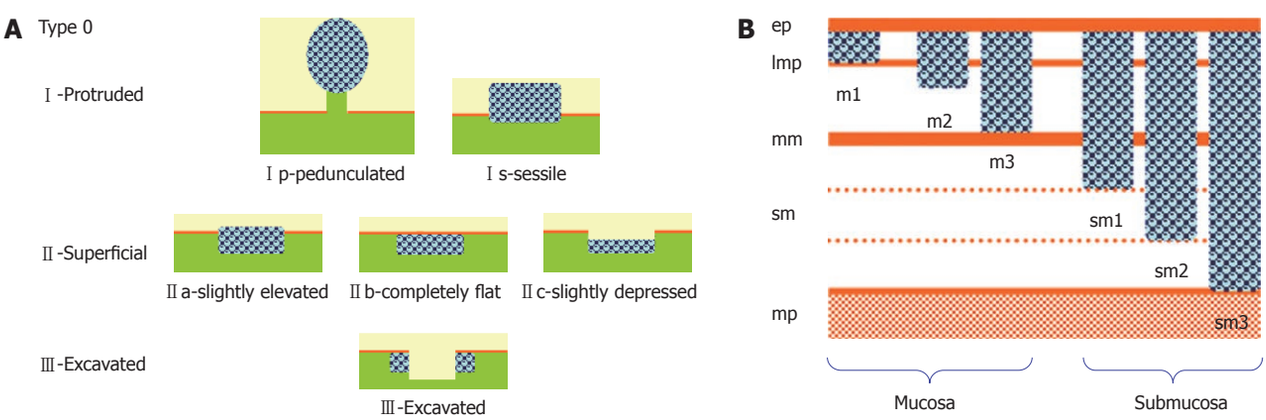

Figure 6 The Paris classification of early lesion of gastrointestinal tract (A) and the depth of tumor infiltration (B). ep: Epithelium; lmp: Lamina propria; mm: Muscularis mucosa; sm: Submucosa; mp:Muscularis propria.

of submucosa; sm2, to the middle third; and sm3, to the lower third. (Figure 6, right side)[69]. The distribution of subtypes in category 0 differs in the esophagus and stomach. As an example, the respective proportions of subtypes 0-Ⅰ and 0-Ⅱc are 16% and 45% in the squamous epithelium of the esophagus, and they are 17% and 78% in the glandular epithelium of the stomach, respectively[70].

Presently, the most critical factor in the decision of whether to perform ESD or *en bloc* EMR is the probability of unexpected lymph node metastasis. Studies have shown that early cancer without lymphovascular involvement could be cured by endoscopic removal. Intromucosal, moderately- or well-differentiated early carcinomas that have been proved do not have submucosal lymphovascular involvement. In contrast, poorly differentiated squamous-cell carcinoma, adenocarcinoma and/or signet-ring cell carcinoma have a high incidence of lymph node metastasis. M1 and m2 carcinomas have no metastasis, whereas less than 10% of m3 carcinomas and about 15%-20% of sm1 have lymph node metastasis. The risk increases to more than 50% of sm2 and sm3 carcinomas[7,67,71,72]. Therefore, before performing ESD or EMR, an accurate histological evaluation of the resected specimens is essential to avoid recurrence.

Magnified images obtained with the ME-NBI system could be a useful, non-invasive method of histologically predicting for early lesions in clinical practice, especially with regard to the IPCL pattern in the esophagus and MV and MS patterns in the stomach, based on which alone usually could help us perform a successful endoscopic therapy. Many researchers focused on the relations between the ME-NBI classifications categories with the characteristics of the histopathological types. For example, regarding the depth of superficial esophageal cancer, the accuracy rate of diagnosis is about 83.3%, according to the Inoue's classification of IPCL[73]. And in the stomach, differentiated-type adenocarcinomas are mainly observed as fine-network patterns in about 15.7% of cases or loop patterns in about 83.8% of cases. Undifferentiated-type lesions are primarily characterized by the corkscrew pattern in approximately 58.8% of cases[57]. For HIN of BE, without the need for staining, the ME-NBI images have a sensitivity of 94% and a specificity of 76% as well as a positively predictive value of 64% and a negatively predictive value of 98%[65].

Comparing the diagnostic accuracy of ME-NBI and endoscopic ultrasonography (EUS) for estimating the depth of invasion of early cancers before removal, some endoscopists conclude that the overall accuracy of ME-NBI is a little higher than EUS, but the difference is not statistically significant. However, ME-NBI is at least as accurate as EUS for preoperative locoregional staging of early cancers. On the other hand, EUS can be used for

observing lymph nodes, but the diagnostic capability of EUS for lymph nodes is less reliable, which can affect therapy-related decisions before ESD. Regarding this point, a consensus is still required. For some cases that are difficult to diagnose, it is even necessary to combine two stool tests with computed tomography before ESD or *en bloc* EMR[74-76].

To define the margin and size of involvement

Criteria for endoscopic submucosal dissection/endoscopic mucosal resection: For differentiated lesions, a size of ≤ 2 cm in diameter is an indication for EMR; a size of ≤ 3 cm of mucosal cancer with ulcers or sm1 submucosal cancers, and any size of mucosal cancer without ulcer are indication for ESD.

In Japan and a few other Asian countries, another current guideline for ESD or *en bloc* EMR regarding well-differentiated lesions is based on data relating the size of the early lesion and the rate of lymph node metastasis. For mucosal cancers with ulcers or sm1 submucosal cancers, lesions that are 3 cm or smaller present a negligible risk of venous or lymphatic involvement. These are indications for ESD. For larger lesions, surgery should be recommended. For lesions confined to the mucosa but without ulcers, the risk of lymph node metastasis is not affected by the size of the tumor, so there is no consensus on a maximal size, although circumferential lesions in the esophagus are usually avoided because of the potential for strictures. Because a 2 cm diameter is the upper limit for resection by EMR in one piece, if the lesions simultaneously meet the conditions of ESD and are not more than 2 cm large, these should also be reasonable indications for *en bloc* EMR treatment because this technique is easier than ESD[6,67,77,78].

Therefore, prior to endoscopic treatment, it is absolutely necessary to accurately identify the full lateral spread of the margins of the lesion, which leads to the determination of the lesion's final size and contributes to the next step of making well-reasoned treatment decisions. In the upper GI, *en bloc* endoscpic removal needs to be carried out 2 mm outside the margin outlined by the spots. This is the key to ensuring that the complete R0 resection has a negative margin for the tumor cells and that the risk of local recurrence is reduced.

ME-NBI allows a more detailed observation of the mucosal changes of microstructures and microvessel patterns of GI carcinoma and is extremely useful, not only for identifying EGC itself, but also for differentiating the borders of cancerous tumors from background non-cancerous mucosa. By ME-NBI, the following points can help determine precise horizontal margins in clinical practice[44]: (1) recognize a demarcation line by the difference between an irregular MV or MS pattern and the surrounding regular normal mucosa. This has been proven to correspond to the tumor margins determined by histopathological examination; and (2) pay close attention to the areas disappearing from the regular SECN pattern as well as the appearance of an ISIV pattern. Sometimes, WOSs are helpful for identifying tumor margins that have not been determined. Also, LBC is a specific indicator for tumors derived from intestinal metaplasia by ME-NBI.

However, for Ⅱb flat reddened lesions that have the same color as the surrounding normal mucosa, it is still occasionally difficult to detect the margins. On the other hand, accurate marking of tumors by ME-NBI also relies on an operator's skill. Therefore, in order to improve the accuracy rate of marking margins, many endoscopists combine ME-NBI with conventional chromoendoscopy. For example, Lugol's solution can dramatically outline the boundaries of a squamous cell esophageal cancer in the esophagus. Although one recent article has concluded that tumor margins can be identified more clearly by ME-NBI than by indigocarmine chromoendoscopy in the stomach[79], it is likely that in the majority of cases, a combination of these two methods prior to ESD or EMR will ensure there are no residual lesions.

To perform a target biopsy in real time

Before local endoscopic *en bloc* resection, the histopathologic diagnosis is very important for making therapy decisions. For a surveillance biopsy to detect early tumors, multiple random biopsies under conventional WL endoscopy are quite time-consuming and may miss a small lesion[80]. For example, for monitoring BE so far, the present recommended strategy is to perform random four-quadrant biopsies at every 2 cm. However, this approach is still prone to sampling errors, inconsistent histopathological interpretations, and delays in diagnosis[81].

Ultimately, the higher-accuracy pathological diagnosis as well as the ultrarapid *in vivo* diagnosis would be preferred in clinical practice[82]. It has been reported that chromoendoscopy could provide a good validity score for early cancer targeted biopsies[83]. However, it still has its limitations, including spending time lost in spraying and washing out the dye. Moreover, some dyes-such as methylene blue-might induce DNA damage in columnar cell-lined mucosa[84].

To this end, in recent years, many researchers suggest using the ME-NBI technique as an "optical biopsy" to better target biopsies in real time. Because this approach can provide better details of mucosa MV and MS patterns that significantly correlate with pathological diagnosis, it has the potential to reduce the need for histological examination of mucosal biopsy specimens[47,49,85,86]. Additionally, some endoscopists even think that ME-NBI can sometimes be substituted for a biopsy before endoscopic therapy because a biopsy might only focus on some suspected, poorly-differentiated lesions under magnifying endoscopy. However, to date, ME-NBI cannot always replace biopsies for histological assessment. In addition, ESD or *en bloc* EMR can supply specimens that are resected in one piece and provide more accurate histopathological diagnosis for determining whether the patient should receive an operation or other treatments[36,87-89].

CONCLUSION

In conclusion, ME-NBI is a very promising endoscopic

technique that can clearly reveal detailed micromorphological differences corresponding to histology and provide some information about layer, origin, size, and extramural extension of GI early lesions. All of these benefits may augment the endoscopic R0 resection of early cancers in the GI tract and help guide targeted biopsies in the surveillance of certain high-risk conditions[19]. To some extent, ME-NBI has now become an indispensable tool in ultra-rapid *in vivo* diagnosis and immediate clinical decision-making, such as when performing ESD or EMR.

In this topic review, most representative references come from the experience of Japanese endoscopists because Japan remains the country with the most ESD cases reported around the world by far. Outside Asia, more recently, techniques such as magnification, NBI and ESD have been increasingly used although viewpoints differ between Eastern and Western cultures, especially regarding extending indications for ESD, the classifications of MV and MS under ME-NBI in the upper GI tract, and partly substituting EUS or biopsy with ME-NBI. However, current data is limited, and we would need long-term outcome data to unify some assessments in order to conduct multicenter trials to develop clear, internationally accepted classification systems. This system review was intended to make a small contribution to some of the aforementioned debates.

Additionally, besides ME-NBI, it is necessary to combine various endoscopic techniques including EUS and chromoendoscopy in some difficult cases before *en bloc* endoscopic resection. It is important to emphasize here that the first step should always be to look carefully for the suspected area by conventional WL endoscopy before switching to the ME-NBI model.

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ORIGINAL ARTICLE

Probiotic modulation of dendritic cells co-cultured with intestinal epithelial cells

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Abstract

AIM: To investigate cytokine production and cell surface phenotypes of dendritic cells (DC) in the presence of epithelial cells stimulated by probiotics.

METHODS: Mouse DC were cultured alone or together with mouse epithelial cell monolayers in normal or inverted systems and were stimulated with heat-killed probiotic bacteria, *Bifidobacterium lactis* AD011 (BL), *Bifidobacterium bifidum* BGN4 (BB), *Lactobacillus casei* IBS041 (LC), and *Lactobacillus acidophilus* AD031 (LA), for 12 h. Cytokine levels in the culture supernatants were determined by enzyme-linked immunosorbent assay and phenotypic analysis of DC was investigated by flow cytometry.

RESULTS: BB and LC in single-cultured DC increased the expression of I-Ad, CD86 and CD40 (I-Ad, 18.51 *vs* 30.88, 46.11; CD86, 62.74 *vs* 92.7, 104.12; CD40, 0.67 *vs* 6.39, 3.37, $P < 0.05$). All of the experimental probiotics increased the production of inflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)-α. However, in the normal co-culture systems, LC and LA decreased the expression of I-A^d (39.46 *vs* 30.32, 33.26, $P < 0.05$), and none of the experimental probiotics increased the levels of IL-6 or TNF-α. In the inverted co-culture systems, LC decreased the expression of CD40 (1.36 *vs* -2.27, $P < 0.05$), and all of the experimental probiotics decreased the levels of IL-6. In addition, BL increased the production of IL-10 (103.8 *vs* 166.0, $P < 0.05$) and LC and LA increased transforming growth factor-β secretion (235.9 *vs* 618.9, 607.6, $P < 0.05$).

CONCLUSION: These results suggest that specific probiotic strains exert differential immune modulation mediated by the interaction of dendritic cells and epithelial cells in the homeostasis of gastrointestinal tract.

Key words: Dendritic cells; Intestinal epithelial cells; Probiotics; Co-culture; Immune modulation

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INTRODUCTION

The gastrointestinal (GI) tract is an immunologic organ

with continuous antigen exposure in the form of food, normal bacteria and pathogens. Despite numerous antigenic challenges, the complicated mucosal immune system maintains GI homeostasis *via* the concerted actions of the various mucosal immune cells. Dendritic cells (DC), dedicated antigen-presenting cells, modulate the immune balance in the GI tract[1]. DC can take up antigens directly by extending their dendrites into the lumen or indirectly after transport of the antigens by M cells overlying Peyer's patch[2,3]. Antigen-carrying DC may traffic through the lymphatics to the mesenteric lymph nodes[4], mediating the homing of activated effector/memory T cells and IgA-secreting B cells[5,6] and inducing regulatory T cells to produce interleukin (IL)-10 and transforming growth factor (TGF)-β[7,8]. These roles depend on the regulation of cell surface expression of co-stimulatory molecules and production of inflammatory chemokines and cytokines[9-11].

DC can recognize and present microbial components using pattern receptor system which includes toll-like-receptor (TLR). TLR can interact with microorganism-associated molecules such as peptidoglycan, lipoprotein, and lipopolysaccharide[12-16]. *Bifidobacterium* and *Lactobacillus* are major components of the commensal microbes of the GI tract and are frequently used as probiotics[17,18]. Probiotics, defined as live microorganisms which, when consumed in appropriate amounts in food, confer a health benefit on the host[19], exert various host physiological responses such as immunomodulatory effect[20]. Recent experiments reported that DC could be modulated by probiotics. Several *Lactobacillus* species could regulate DC surface expression and cytokine production[21]. In addition, the probiotics mixture VSL No. 3 upregulated the expression of major histocompatibility complex (MHC) class Ⅱ and co-stimulation molecules[22].

DC are often located close to epithelial cells, populating the subepithelial dome of Peyer's patches, immediately adjacent to the follicle-associated epithelium and the lamina propria[23,24]. Intestinal epithelial cells secrete many mediators, including functional peptides such as defensins, mucins, chemokines, and cytokines such as IL 8[25-27]. TLR5 on the epithelium is a key mediator of pro-inflammatory responses to flagella from commensal bacteria[28,29]. Flagella also stimulate the maturation of responsive DC[30].

Interaction between DC and epithelial cells is integral to the intestinal immune system. We hypothesized that epithelial cells stimulated by probiotics could regulate the maturation of DC. Accordingly, the present study investigated the pattern of cytokine production and the surface phenotype of DC in the presence of epithelial cells polarized by heat-killed probiotic bacteria.

MATERIALS AND METHODS

Preparation of probiotic bacteria

Bifidobacterium bifidum BGN4 (BB) was isolated from healthy infant fecal matter and identified in our laboratory[31]. *Bifidobacterium lactis* AD011 (BL), *Lactobacillus casei* IBS041 (LC), and *Lactobacillus acidophilus* AD031 (LA) were provided by the Research Institute of Bifido Co. Ltd. (Hongchun, Gangwondo, South Korea). Four probiotic bacteria were anaerobically propagated in de Man, Rogosa, and Sharpe (Difco, Detroit, MI, United States) broth containing 0.05% L-cysteine (Sigma, St. Louis, MO, United States) at 37 ℃ until mid-log phase was reached. Subsequently, probiotics were inoculated at 1% and anaerobically cultured in de Man, Rogosa, and Sharpe (Difco) broth containing 0.05% L-cysteine (Sigma) at 37 ℃. *Lactobacillus* species were incubated for 16 h, and *Bifidobacteirum* species were incubated for 24 h to late log phase. The bacteria were collected by centrifugation at 1000 × *g* for 15 min at 4 ℃ and washed twice with phosphate-buffered saline (PBS). After washing, the bacteria were resuspended in 1 mL of PBS and incubated at 95 ℃ for 30 min to prepare heat-killed bacteria cells. The killed bacteria were collected by centrifugation at 1000 × *g* for 15 min and then lyophilized (Combi-514R, Hanil Science Industrial, Seoul, South Korea).

Generation of CMT-93 monolayers

CMT93 was derived from carcinomas of C57BL mouse large intestine. The cells have an epithelial morphology and forms acini, junctional complexes, and microvilli with attached glycoprotein[32]. CMT-93 cells were maintained in DMEM (Gibco Life Technologies, United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, United Kingdom) and 1% penicillin/streptomycin (Invitrogen), and were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. Monolayers were grown in 24-well Corning Costar Transwell plates (Corning Inc., United States) with 3 μm pore-size filter inserts. In the normal co-culture system, 5×10^5 cells were seeded into the inserts, and the wells were filled with 1 mL medium. In the inverted co-culture system, inserts were removed and inverted in tissue culture dishes, and the cells of the same volume were seeded to the exposed filter membrane. The culture dishes were filled with enough medium to sink the inserts. The transwell inserts were cultured for 3-4 d until CMT-93 established monolayers. Confluence of the cells was confirmed when the trans-epithelial electrical resistance (TEER; Millicell ERS Ohmmeter, Millipore, Eschborn, Germany) exceeded the cut-off point of 250 Ω/cm^2.

JAWS Ⅱ cell preparation

JAWS Ⅱ, mouse bone marrow-derived immature DC[33], were maintained in α-MEM (Gibco) supplemented with 5 ng/mL GM-CSF (Sigma, St. Louis, MO, United States), 20% heat-inactivated FBS (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The mixture was incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. The cells were cultured at a 1/2 subcultivation ratio for 5-6 d in complete medium.

Co-culture experiment model

The co-culture experiment model is shown in Figure 1. JAWS Ⅱ cells were harvested, washed, and resuspended in RPMI1640 complete medium (Gibco) containing 5 ng/mL GM-CSF (Sigma), 10% heat-inactivated FBS

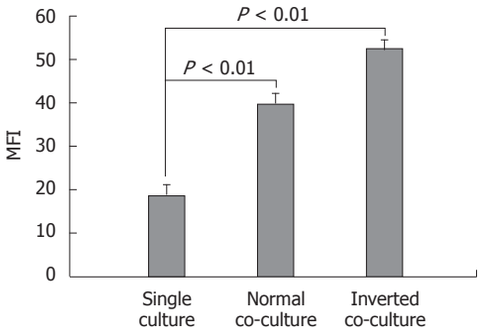


Figure 1 Effect of non-stimulated intestinal epithelial cells on surface phenotype of dendritic cells. Fluorescence activated cell sorter analysis of dendritic cells (DC) cultured alone or co-cultured with non-stimulated epithelial cell monolayers for 12 h showing DC surface phenotype by staining with I-A^d. Data are shown as the mean fluorescent intensity (MFI) ± SEM of three representative experiments. Significant difference between the single culture and co-culture as determined by Student's *t*-test ($P < 0.01$).

(Invitrogen), and 1% penicillin/streptomycin (Invitrogen). A total of 1×10^6 JAWS Ⅱ cells were added into lower chambers, and the normal and inverted cultured CMT-93 monolayer inserts were placed in the JAWS Ⅱ seeded transwell plates. One hundred μg/mL of the experimental bacteria or 10 μg/mL of LPS (Sigma) were added to the CMT-93 monolayer inserts. For comparison, JAWS Ⅱ cells were also plated at the same concentration in 24 well tissue culture plates (Corning Inc.), and the same amount of the bacteria or LPS were added to the cells. The single- or co-cultured cells were incubated with 1 mL RPMI1640 complete medium at 37 ℃ in a humidified atmosphere of 5% CO_2 for 12 h.

Flow cytometry analysis

Incubated JAWS Ⅱ cells were harvested and washed three times in cold FACS buffer (Dulbecco's PBS; Gibco, 2% FBS) and then stained with the appropriate monoclonal antibodies: PE-conjugated anti-I-A^d, anti-CD80, anti-CD86, and anti-CD40 at a final concentration of 10 μg/mL for 30 min at 4 ℃ in the dark. Isotype control antibodies were hamster IgG2 k, rat IgG2a k, and mouse IgG2b. The stained cells were analyzed immediately by FACSCalibur (Becton Dickinson, San Diego, CA, United States). All of the antibodies used in this flow cytometry analysis were purchased from Pharmingen (San Diego, CA, United States).

Cytokine measurement

JAWS Ⅱ cell supernatants were harvested from the lower chamber of the Transwell or from the JAWS Ⅱ cultured-alone plate following incubation, and were assayed for levels of IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF)-α and TGF-β using enzyme-linked immunosorbent assay . Briefly, Nunc-Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 2 μg/mL of rat anti-mouse IL-6 and TGF-β capture antibodies in coating buffer (1.6 g/L Na_2CO_3, 7.1 g/L $NaHCO_3$), pH 9.5, or 2 μg/mL of rat anti-mouse IL-10, IL-12p70, and TNF-α capture antibodies in coating buffer (11.8 g/L Na_2HPO_4, 16.1 g/L NaH_2PO_4), pH 6.5, overnight at 4 ℃. After washing and blocking, 100 μL of 1:100 diluted (IL-6) or undiluted (IL-10, IL-12p70, TNF-α and TGF-β) supernatant was added to individual wells and incubated overnight at 4 ℃. Plates were washed, and biotinylated rat anti-mouse IL-6, IL-10, IL-12p70, TNF-α and TGF-β monoclonal antibodies (2 μg/mL) and HRP-conjugated streptavidin were added to the plates for cytokine detection for 1 h at room temperature. The reactions were developed with the 3,3',5,5'-tetramethylbenzidine substrate (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 2 N H_2SO_4 and analyzed at 450 nm. Equivalent levels of IL-6, IL-10, IL-12p70, TNF-α and TGF-β were measured for comparison with a reference curve generated using standards of these cytokines.

Statistical analyses

Data are presented as the mean ± SE, indicated by bars in the figures. All statistical analyses were performed using SPSS 12.0K for Windows (SPSS Inc., Chicago, IL, United States). Differences between the single culture and co-culture were determined by Student's t-test, and differences between cytokine levels were analyzed by analysis of variance followed by Duncan's multiple range test. The *P* values < 0.05 were considered to be statistically significant.

RESULTS

Development of stable CMT-93 epithelial cell monolayers

To obtain stable CMT-93 intestinal epithelial cell monolayers, we monitored the culture every day for TEER using a Millicell-ERS ohmmeter for a period of 7 d. On day 3, normal insert monolayer integrity was obtained at 300-500 Ω/cm^2, and inverted insert monolayer integrity was obtained at 250-350 Ω/cm^2. In addition, the generation of epithelial cell monolayers was observed on the surface of the inserts by microscope (data not shown). Monolayers between day 3 and 4 were used for co-culture experiments. After co-culture with DC for 12, the integrity of CMT-93 monolayer was evaluated by TEER. There was no difference between before and after co-culture in terms of the resistances within the margin of error.

Dendritic cells phenotype modulation during co-culturing with epithelial cells

DC surface phenotypes were compared in the presence and absence of epithelial cells. The expression of MHC class Ⅱ I-A^d on the normal and the inverted co-cultured DCs was upregulated compared with that of the single-cultured DC (single culture, 18.51 ± 2.86; normal co-culture, 39.46 ± 2.53; inverted co-culture, 52.03 ± 2.41; Figure 1). Co-culture with epithelial cells did not alter the DC surface expression of CD80, CD86 and CD40 (data not shown).

Effect of probiotics on the expression of major histocompatibility complex class Ⅱ and costimulatory molecules

We performed flow cytometery analyses to examine the

effects of BL, BB, LC, LA, LPS and control on single- or co-cultured immature DC surface phenotypes. In the DC single culture, the expression of MHC class Ⅱ I-A^d was significantly increased by stimulation with BL, BB, and LC compared with the control (Figure 2). In the normal co-culture, the expression of I-A^d was significantly decreased by the stimulation of LC and LA compared with the control. In the inverted co-culture, none of the experimental probiotics modulated the expression of I-A^d.

BL and LA significantly downregulated the expression of CD80 in the single-cultured DC. However, none of the experimental probiotics regulated CD80 in the normal and inverted co-cultured DC (Figure 3A).

BB and LC upregulated the expressions of CD86 and CD40 in the single-cultured DC, whereas none of the experimental probiotics regulated the expression of CD86 in the normal or inverted co-cultured DC (Figure 3B). LC significantly downregulated the expression of CD40 in the inverted co-cultured DC compared with medium alone (Figure 3C).

Cytokine profiles in dendritic cells supernatant by co-culturing with epithelial cells

The levels of IL-6, IL-12p70, TNF-α and TGF-β from the co-cultured system were significantly reduced compared with those from the single-cultured DC (Figure 4A and C-E); however, the production of IL-10 showed no decrease in the co-cultured DC.

Effect of probiotics on the cytokine production in the co-culture system

We quantified the cytokine levels in the single- and co-cultured DC supernatants to investigate the effect of the experimental probiotics on the production of cytokines. LPS was used as a stimulator control to compare with non-treated naïve control. In the single-cultured DC, stimulation with the experimental probiotic bacteria markedly increased the production of IL-6 and TNF-α compared with the control (Figure 4A and D). In the normal co-cultured DC, the levels of IL-6 stimulated by BB and LC and the level of TNF-α stimulated by LA were lower than those of non-stimulated control. In the inverted co-cultured DC, all of the experimental bacteria significantly decreased the production of IL-6 compared with the control, but had no significant effect on the production of TNF-α.

In the single-culture, the level of IL-10 in DC stimulated by BL, LC,and LA was higher than that in the control DC (Figure 4B). The levels of IL-10 from the normal co-cultured DC stimulated by BL, BB and LC were lower. IL-10 from the inverted co-cultured DC stimulated by BL was higher than that from the control.

In the single-cultured DC all of the experimental probiotics decreased the production of IL-12p70. In the normal co-cultured DC only BL increased the production of IL-12p70. In the inverted co-cultured DC the levels of IL-12p70 stimulated by all of the experimental probiotics were similar to that of control.

BL, BB and LC in the single-cultured DC decreased the production of TGF-β. The levels of TGF-β in all of the treated groups in the normal co-culture system were similar to that from the non-stimulated control but in the inverted co-cultured system LC and LA significantly increased the production of TGF-β (Figure 4C and E).

DISCUSSION

The modulatory effect of probiotics on the host immune system was reported in *in vivo* experiments and clinical trials[34]. However, the exact mechanism of the immunomodulatory effect of probiotics, especially with respect to the interaction between DC and epithelial cells in the presence of probiotics, has not been well elucidated. In the present study, we investigated the effect of heat-killed BL, BB, LC and LA on the modulation of JAWS Ⅱ (DC) using an *in vitro* co-culture model. *In vitro*, live bacteria grown by geometric progression exhausted culture nutrients, produced various acid metabolites, and induced necrosis of the cultured animal cells within a few hours. Therefore, the treatment of the host cells with live bacteria was inappropriate. The adhesive properties of heat killed bacteria might be differentially modified by the heat treatment depending on the specific strain of the experimental bacteria. However, a previous study reported that oral administration of heat-killed and lyophilized BB could suppress the occurrence of allergy by the immune regulatory actions in the mouse allergy model[35], which implied that heat-killed and lyophilized bacteria could maintain their immunomodulatory effects.

To simulate the interaction between DC and epithelial cells in the intestinal environment, we used a transwell co-culture system with CMT93 epithelial cell lines[32]. CMT93 was derived from the same mouse origins as JAWS Ⅱ, C57BL mouse, and forms junction complexes. A previous study reported that there was a gap junctional communication between murine lymphocytes and CMT93 epithelial cells, and gap junctional communication might regulate cell functions[36]. Nonpathogenic intestinal bacteria can induce DC migration into the epithelial layer and recruit DC uptake of bacteria and apoptotic fragments derived from apoptotic epithelial cells to maintain peripheral self-tolerance[2,37]. In the normal co-culture system, there was an insert membrane and a gap of 1 mm between JAWS Ⅱ and CMT93. On the other hand, an inverted co-culture model was established by the generation of a CMT93 monolayer on the underside of the inverted insert. A previous study demonstrated that DCs directly interacted with luminal bacteria using CX_3CR1-mediated trans-epithelial dendrites[38].

DC interact with microbes and distinguish gram positive, negative, or closely related organisms using TLR and present the processed antigens through MHC class Ⅱ[12,16,39]. DC then mediate T cell activation, which is regulated by MHC class Ⅱ molecules, co-stimulatory molecules such as CD80 and CD86, and cytokines[40].

In the single-cultured systems BB and LC upregulated the expression of MHC class Ⅱ I-A^d, CD86, and CD40, while all of the experimental probiotics attenuated IL-

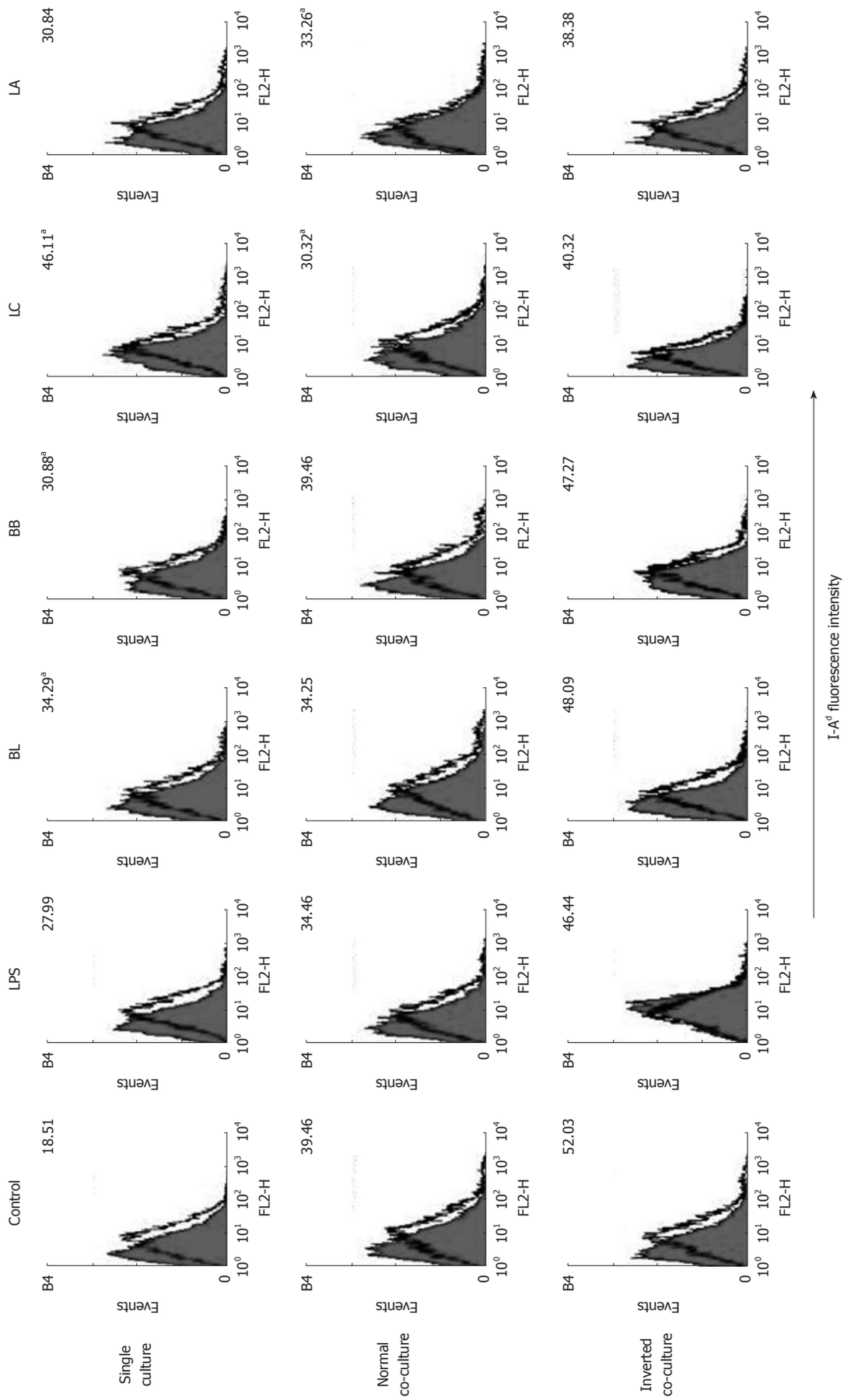
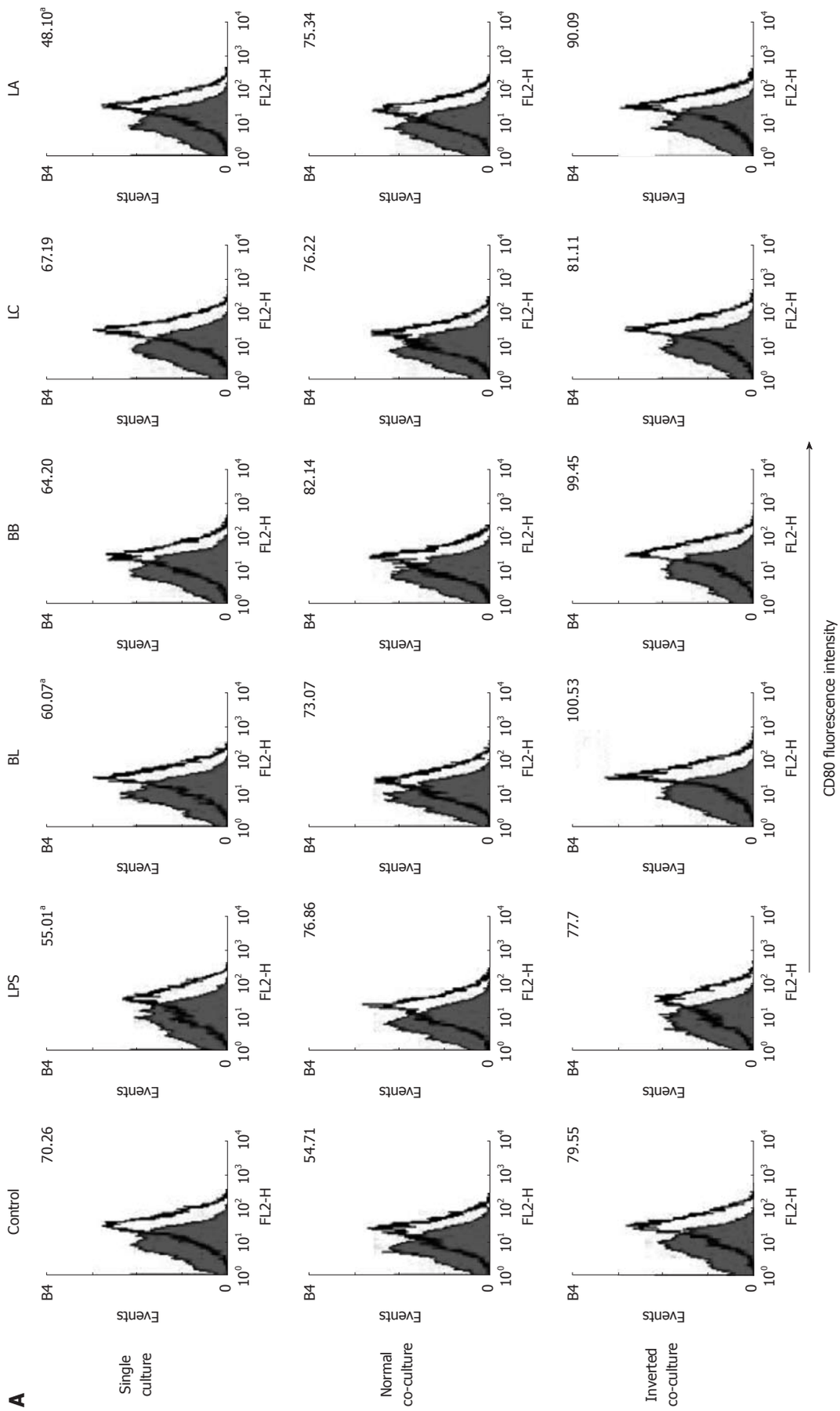


Figure 2 Effect of probiotics on I-A^d of single- or co-cultured dendritic cells. Fluorescence activated cell sorter analysis of probiotics-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Filled histograms are isotype controls; unfilled histogram show staining for I-A^d. Numbers indicate the mean fluorescent intensity of three representative experiments. ^{a}Significant difference among the control, lipopolysaccharides and probiotics as determined by analysis of variance ($P < 0.05$). LPS: Lipopolysaccharides; BL: *Bifidobacterium lactis* AD011, BB: *Bifidobacterium bifidum* BGN4, LC: Lactobacillus casei IBS041; LA: Lactobacillus acidophilus AD031.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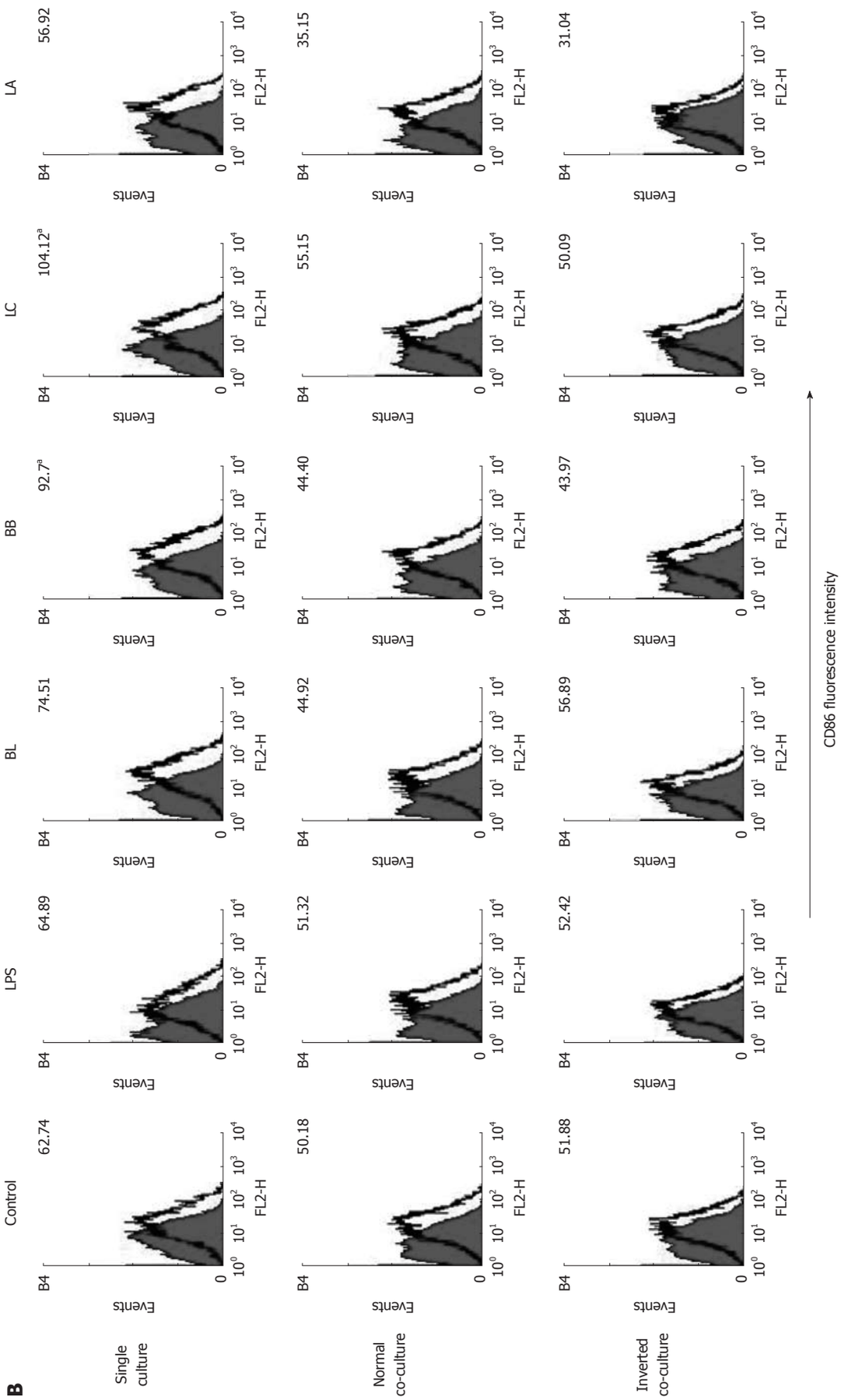
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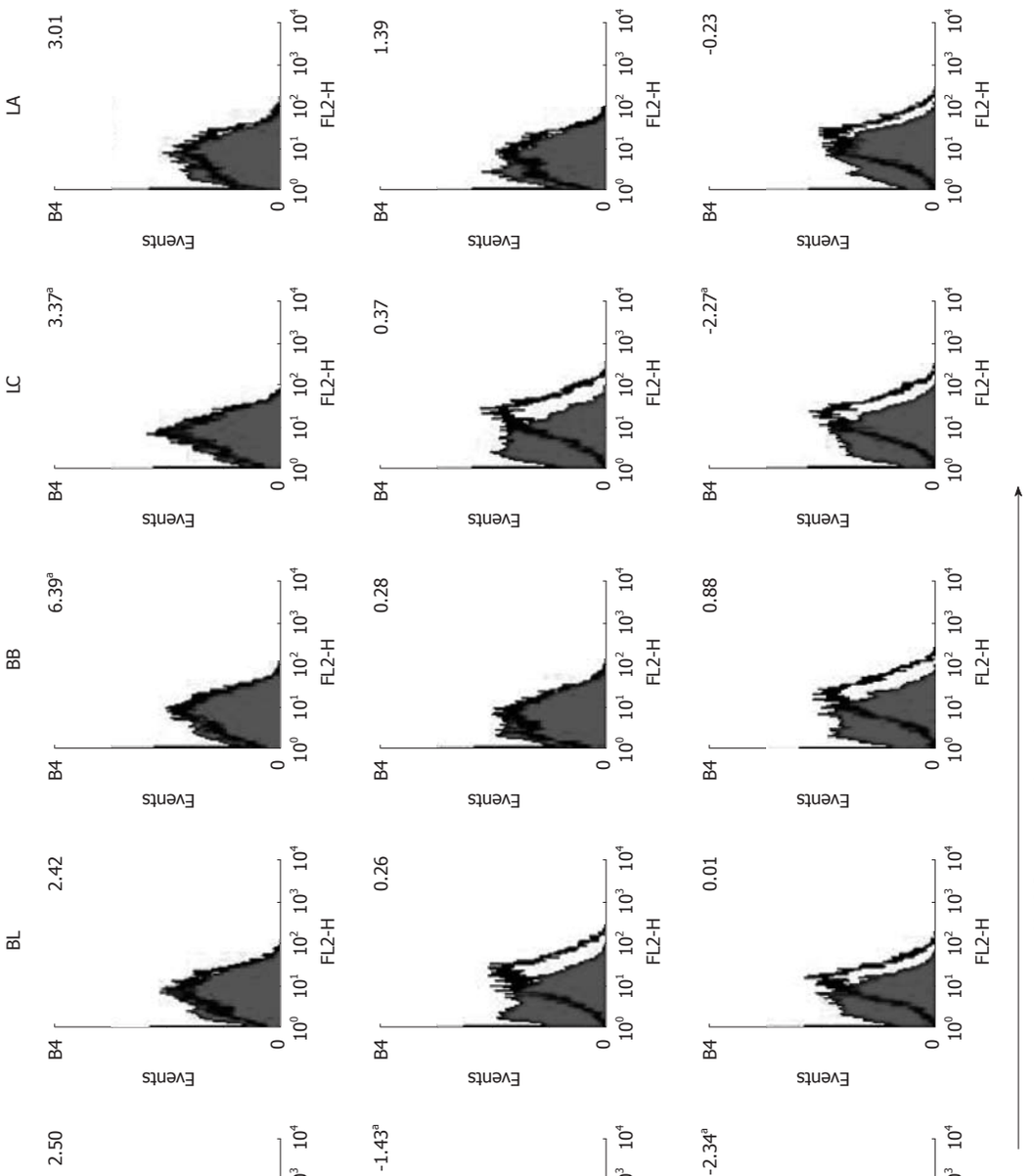

Figure 3 Effect of probiotics on the CD80, CD86 and CD40 of single- or co-cultured dendritic cells. Fluorescence activated cell sorter analysis of probiotics-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Filled histograms are isotype controls; unfilled histogram shows staining for CD80 (A), CD86 (B) and CD40 (C). Numbers indicate the mean fluorescent intensity of at least three representative experiments. [a]Significant difference among the control, lipopolysaccharides, and probiotics as determined by analysis of variance ($P < 0.05$). LPS: Lipopolysaccharides; BL: *Bifidobacterium lactis* AD011, BB: *Bifidobacterium bifidum* BGN4, LC: Lactobacillus casei IBS041; LA: Lactobacillus acidophilus AD031.

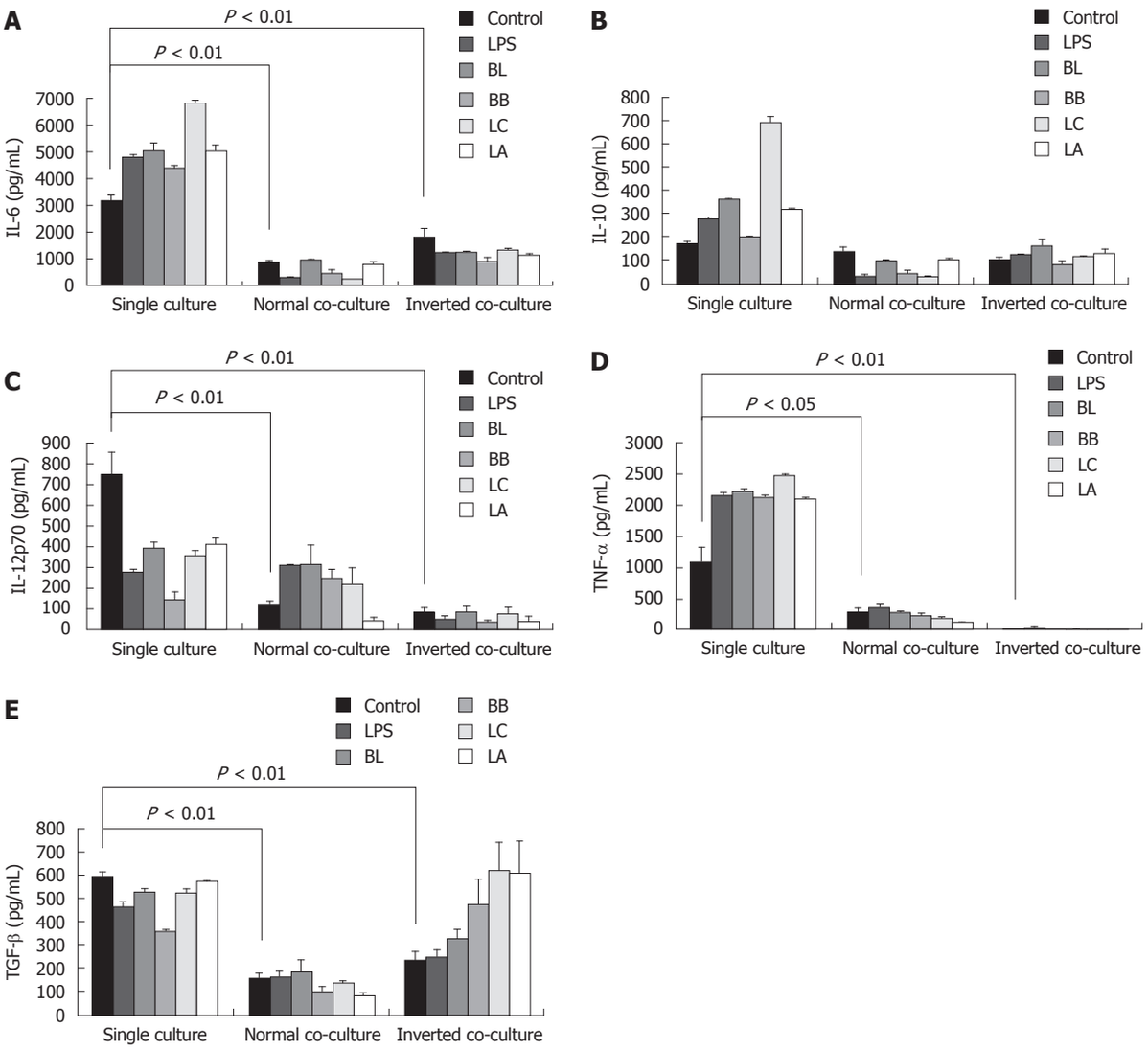

Figure 4 Effect of probiotics on the production of cytokines from single- or co-cultured dendritic cells. Supernatants were obtained from probiotic-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Levels of interleutkin (IL)-6 (A), IL-10 (B), IL-12p70 (C), tumor necrosis factor (TNF)-α (D), and transforming growth factor(TGF)-β (E) were determined by enzyme-linked immunosorbent assay. Data are shown as mean ± SE of three representative experiments. Different letters indicate significant differences among the control, lipopolysaccharides (LPS), and probiotics determined by Duncan's multiple range test ($P < 0.05$). Significant difference between the single culture and co-culture as determined by Student's *t*-test ($P < 0.05$). BL: *Bifidobacterium lactis* AD011; BB: *Bifidobacterium bifidum* BGN4; LC: *Lactobacillus casei* IBS041; LA: *Lactobacillus acidophilus* AD031.

12p70 secretion. IL-12 directed the differentiation of T cells to a Th1 phenotype[41]. Nier reported that *Bifidobacterium bifidum* enhanced the expression of CD86 and MHC class Ⅱ in human neonatal DC, which led in turn to the polarization of IFN-γ-producing T cells[42]. Mohamadzadeh *et al*[43] showed that *Lactobacillus gasseri, Lactobacillus johnsonni*, and *Lactobacillus reuteri* upregulated the expression of MHC class Ⅱ, CD40, CD80 and CD86 in human myeloid DC and increased the level of IL-12p70 which induced the polarization from CD4(+) and CD8(+) T cells to T helper 1 and Tc1 cells. Meanwhile, Drakes *et al*[22] showed that probiotic products containing *Lactobacillus* and *Bifidobacterium* upregulated the expression of MHC class Ⅱ, CD40, CD80 and CD86, and did not induce the production of IL-12p70 in mouse DC. Additionally, mouse bone marrow-derived DC treated with *Lactobacillus reuteri* induced Th2 immune response[21]. Taken together, the results of these earlier studies suggested that probiotics upregulated the expression of MHC class Ⅱ and differently modulated co-stimulatory molecules such as IL-12p70 and T cell polarization, depending on the DC origin and the strain of probiotics.

Interestingly, in the present study the effects of probiotics on cytokine production and the surface phenotype in co-cultured DC with epithelial cells were markedly different from those in single-cultured DC. All of the experimental probiotics induced the production of pro-inflammatory cytokines, IL-6 and TNF-α, in the single cultured DC. TNF-α mediated various immune responses[44], and over-production of TNF-α could play a role in tissue damage and intestinal pathologies[45,46]. In contrast with the results from the single system the experimental probiotics reduced or did not affect the expression of I-A^d, CD86 and CD40 or the production of IL-6, IL-12p70

and TNF-α in inverted co-cultured DC. These findings suggest that epithelial cells are essential components of the immune system to be considered in assessing the effects of probiotics on the regulation of the gastrointestinal immune system. Consequently, previous studies which employed only DC cells without epithelial cells might have provided only partial pictures or sometimes misleading information about the interaction of the probiotics with DC cells.

Previously, Haller *et al*[47] showed that *Lactobacillus johnsonii* increased the production of TGF-β in human epithelial cell lines co-cultured with leucocytes. In our study, BL, LC and LA induced IL-10 secretion from single-cultured DC. In an inverted co-culture system, BL increased IL-10 secretion, and LC and LA increased TGF-β secretion. IL-10 was known to activate regulatory T cells[48]. TGF-β which is an important factor in enhancing the differentiation of regulatory Th3 cells was reported to have wide-ranging immunomodulatory properties[49,50]. Th3 cells suppress Th1 and other immune responses and maintain oral tolerance[40,50,51]. Conceivably, enhanced secretion of IL-10 or TGF-β observed in the co-culture systems by BL, LC and LA might contribute to the activation of regulatory T cells in the intestinal tracts. The present study is novel since we assessed the effect of probiotics on immunemodulation in a co-culture model. We suggest that a co-culture model better reflects the environmental status of the *in vivo* immune system. Our model supports the hypothesis that the interaction of DC and epithelial cells stimulated with probiotics may help maintain intestinal homeostasis by downregulating the production of inflammatory cytokines and expression of MHC class Ⅱ in DC.

COMMENTS

Background

It is known that dendritic cells (DCs) modulate the immune balance in the intestinal tract by mediating the activation of different subsets of T cells. The functions and differentiation of the DCs may be modulated by probiotics. To better understand the role of the probiotics in the intestinal immune system the interactions of probiotics in the context of epithelial cells and DCs need to be assessed.

Research frontiers

Intestinal epithelial cells secrete various immunological mediators, but the interactions between probiotics, intestinal epithelial cells, and DCs were not fully known. The present study investigated the pattern of cytokine production and the surface phenotype of DCs in the presence of epithelial cells polarized by heat-killed probiotic bacteria.

Innovations and breakthroughs

The present study showed the differential effects of probiotics between the single cultured and the co-cultured DCs. We suggest that a co-culture model better reflects the environmental status of the *in vivo* immune system. The interaction of DCs and epithelial cells polarized with probiotics may contribute to the homeostasis of the immune system in the intestinal tracts.

Applications

The employment of the co-cuture system may facilitate the development of probiotic bacteria with immunomodulatory effects for people with hypersensitive or imbalanced immune symptoms.

Peer review

The study is well-conducted and results are interesting. However, the results are curiously reported in a confusing manner. Moreover, some controls are needed and the positive control used should be more justified.

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ORIGINAL ARTICLE

Enhancement of CTLs induced by DCs loaded with ubiquitinated hepatitis B virus core antigen

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Abstract

AIM: To investigate whether hepatitis B virus (HBV) could induce a hepatitis B virus core antigen (HBcAg)-specific cytotoxic T lymphocyte (CTL) response *in vitro* by dendritic cells (DCs) transduced with lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg).

METHODS: Recombinant LV-Ub-HBcAg were transfected into highly susceptible 293 T cells to obtain high virus titres. Bone marrow-derived DCs isolated from BALB/c mice were cultured with recombinant granulocyte-macrophage colony-stimulating factor and recombinant interleukin (IL)-4. LV-Ub-HBcAg, lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg), lentiviral vector (LV) or lipopolysaccharide were added to induce DC maturation, and the DC phenotypes were analyzed by flow cytometry. The level of IL-12 in the supernatant was detected by enzyme-linked immunosorbent assay (ELISA). T lymphocytes were proliferated using Cell Counting Kit-8. DCs were cultured and induced to mature using different LVs, and co-cultured with allogeneic T cells to detect the secretion levels of IL-2, IL-4, IL-10 and interferon-γ in the supernatants of T cells by ELISA. Intracellular cytokines of proliferative T cells were analyzed by flow cytometry, and specific CTL activity was measured by a lactate dehydrogenase release assay.

RESULTS: LV-Ub-HBcAg-induced DCs secreted more IL-12 and upregulated the expression of CD80, CD86 and major histocompatibility class Ⅱ. DCs sensitised by different LVs effectively promoted cytokine secretion; the levels of IL-2 and interferon-γ induced by LV-Ub-HBcAg were higher than those induced by LV-HBcAg. Compared with LV-HBcAg-transduced DCs, LV-Ub-HBcAg-transduced DCs more efficiently stimulated the proliferation of T lymphocytes and generated HBcAg-specific cytotoxic T lymphocytes.

CONCLUSION: LV-Ub-HBcAg effectively induced DC maturation. The mature DCs efficiently induced T cell polarisation to Th1 and generated HBcAg-specific CTLs.

Key words: Ubiquitin; Hepatitis B virus core antigen; Lentiviruses; Dendritic cells; Cytotoxic T lymphocytes

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem, particularly in Asia and South Africa[1]. Notably,

an effective T cell response is critical for virus clearance, and defective cytotoxic T lymphocytes (CTLs) may lead to persistent HBV infection[2]. Moreover, the defective CTL response was ascribed to the impaired dendritic cell (DC) function[3]. Promoting and improving DC function is a promising approach to combating persistent HBV infection.

Various methods have been attempted to modify DC function, including the use of protein antigens, cytokines, costimulatory molecules, and signalling pathway ligands known to activate the immune response[4-7]. Nevertheless, these methods may be insufficient to induce a strong antigen-specific immune response. Further enhancement of the immune response to HBV-specific CTL may be more conducive to clear the HBV. Thus, a novel therapeutic approach is needed to activate T cell expansion and induce a strong antigen-specific T cell response.

Ubiquitin (Ub) is a highly conserved small regulatory protein, ubiquitous in eukaryotes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes[8]. The Ub-mediated processing of antigens is rapid and efficient and stimulates cell-mediated immune responses. Accordingly, Ub-mediated processing of antigens has been widely used in chronic infection and cancer studies to improve immune response. Wang *et al*[9] found that an Ub-fused *Mycobacterium tuberculosis* antigen ESAT-6 DNA vaccine significantly increased the antigen-specific cellular immune response in BALB/c mice. That study confirmed that the Th1-type immune response and CTL activity were enhanced by changing the antigen processing. Zhang *et al*[10] reported that Ub-fused melanoma antigens induced antigen proteins to execute proteasome-dependent degradation and created epitopes of major histocompatibility complex (MHC) class I , resulting in the preferential activation of antigen-specific $CD8^+$ T cells.

Retroviral and adenoviral vectors have been the focus of many studies because of their high efficiency. Lentivirus vectors (LVs) transfect both dividing and relatively quiescent cells and have been widely used to modify DCs[11,12]. The aim of this study was to investigate the capacity of DCs transfected with LVs encoding the ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg-DC) to stimulate lymphocyte proliferation and to generate antigen-specific CTLs. The results may provide effective approaches to the control of persistent HBV infection.

MATERIALS AND METHODS

Animals

BALB/c mice ($H\text{-}2^d$), 6-8 wk old, were purchased from the Shanghai Experimental Animal Centre of the Chinese Academy of Sciences and maintained under pathogen-free conditions. Mice were cared for and treated in accordance with the guidelines established by the Shanghai Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Cell lines

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Gaithersburg, MD, United States) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, United States), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 ℃ in 5% CO_2. The $H\text{-}2^d$ mastocytoma cell line P815/c (expressing the HBV core antigen) was maintained in our lab.

Construction of lentiviral vectors

The plasmid pcDNA3.1(-)-Ub-HBcAg was constructed and maintained in our lab. The *Ub-HBcAg* gene was amplified by polymerase chain reaction (PCR). The primers used were: Ub-HBcAg: forward: CGTGGGATCCATGCA GATCTTCGTGAAG, reverse: CGCACG CGTCTAACATTGAGATTCCCGAG from plasmid pcDNA3.1(-)-Ub-HBcAg. The purified Ub-HBcAg fragment was cloned into the pWPLXd vector (provided by Prof. Jianming Li, Nanjing, China) using *Bam*H I and *Mlu* I restriction sites. The recombinant pWPLXd- Ub-HBcAg plasmid was confirmed by restriction enzyme digestion and DNA sequencing. LV-Ub-HBcAg was derived by a combined transfection of three elements: 10 μg pWPLXd-Ub-HBcAg backbone plasmid, 5 μg psPAX2 packaging plasmid, and 5 μg PMD2.G envelope plasmid. We transiently transfected 293T cells with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States). Two days after the transfection, the viral supernatant was collected and filtered through a 0.45-μm filter. Concentrated vectors for the *in vitro* studies were prepared by ultracentrifugation at 25 000 rpm and 4 ℃ for 90 min. Viral pellets were resuspended in 2 mL sterile phosphate-buffered saline (PBS) and stored at -80 ℃.

The control plasmid was constructed by inserting the HBcAg fragment into the *Bam*H I and *Mlu* I site of the pWPLXd plasmid and named pWPLXd-HBcAg. LV particles (LV-HBcAg) were produced by Lipofectamine transfection into 293T cells.

To determine the titre of the green fluorescent protein (GFP)-expressing vector, 293T cells (1×10^6 cells/well) were infected with serially diluted viral supernatant. On day 2, the infected cells expressing GFP were counted by flow cytometry. The titre was calculated as: transduction units per mL (TU) = the number of infected cells/volume of virus supernatant.

Western blotting

The 293T cells were seeded in six-well plates at 1×10^6 cells/well. LV-Ub-HBcAg, LV-HBcAg or LV was added at an multiplicity of infection (MOI) of 1. In some experiments, a specific inhibitor of proteasomes, MG132, was used at 10 μmol. The cells were harvested 48 h after infection, washed twice with PBS, gently dispersed into a single-cell suspension and homogenised using RIPA lysis buffer. Protein concentrations were determined using the Pierce BCA Protein Assay Reagent kit (Rockford, IL, United States). Homogenates were diluted to the desired protein concentration with 2 × SDS-PAGE loading buffer (Invitrogen). Samples were boiled and loaded onto polyacrylamide mini-gels (Invitrogen) for electrophoresis. Proteins from the gels were transferred to Im-

mobilon-PVDF membranes (Millipore Corp., Bedford, MA, United States) using a semi-dry apparatus (Bio-Rad, Hercules, CA, United States). A mouse anti-human HBcAg monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) was used as the primary antibody, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin-G antibody was used as the secondary antibody.

Dendritic cell generation

Femurs and tibiae of Babl/c mice were removed and purified from the surrounding muscle tissues. Thereafter, intact bones were left in 70% ethanol for 5 min for disinfection and then washed with PBS. Both ends were cut with scissors and the marrow was flushed with PBS using a syringe with a 0.45-mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting. Bone marrow cells were cultured at 2×10^6 cells/mL in complete RPMI 1640 culture medium (containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin) in the presence of 20 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, United states) and 10 ng/mL murine IL-4 (mIL-4; PeproTech). Nonadherent single cells were gently removed, and fresh medium containing murine GM-CSF and mIL-4 was added on day 3 after beginning culture.

Dendritic cell immunophenotyping

On day 5, immature DCs were cultured for an additional 96 h in the presence of LV-Ub-HBcAg, LV-HBcAg or LV (MOI = 20), and lipopolysaccharide (LPS, 0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) was used as a control group. On day 9, non-adherent and loosely adherent cells were harvested as DCs. The expression of DC surface molecules was analyzed by incubation with allophycocyanin-labelled anti-mouse CD11c, CD80, CD86 and MHC class Ⅱ (eBioscience, San Diego, CA, United States). The stained cells were analyzed by flow cytometry.

Interleukin-12 production

On day 5, immature DCs were infected with LV-Ub-HBcAg, LV-HBcAg, or LV (MOI = 20) for 72 h. On day 8, the IL-12 levels in harvested supernatants of mature DCs were measured using a standard sandwich enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions.

Mixed leukocyte reaction

On day 9, harvested mature DCs were pre-treated with 25 μg/mL mitomycin C and 5% CO_2 for 30 min at 37 ℃. Mouse spleens were dissociated on 200-gauge nylon mesh. Splenocytes were collected and treated with lysis buffer to eliminate red cells, washed, and resuspended in RPMI-1640 with 10% FBS. Lymphocytes were derived from splenocytes using nylon wool columns. Single-cell suspensions of lymphocytes (5×10^5 cells/well) were grown in 96-well plates. Lymphocytes were co-cultured with mature DCs at different responder/stimulator (T cell/DC) ratios (5:1, 10:1 or 20:1) for 72 h. The cells were incubated in a final volume of 200-μL complete RPMI 1640 for 72 h, and 10-μL Cell Counting Kit-8 solution (Beyotime Institute of Biotechnology, Haimen, China) was added to the plates for 4 h at 37 ℃. The absorbance was finally read at 450 nm.

Cytokine production

Splenocytes from mice were cultured in 96-well culture plates in the presence of mature DCs for 4 d at a T-cell to DC ratio of 10:1, and the supernatants were collected. The levels of different cytokines [interferon (IFN)-γ, IL-2, IL-4 and IL-10] in the supernatants of proliferating T cells were measured using commercial ELISA kits according to the manufacturer's protocol (R and D Systems). Data were expressed as pg/mL.

IFN-γ production was detected by intracellular staining and flow cytometry. The above proliferative T cells were suspended in complete RPMI 1640 and stimulated for 6 h in the presence of 25 μg/mL phorbol 12-myristate 13-acetate, 1 μg/mL ionomycin and 1.7 μg/mL monensin (Sigma). After washed with PBS, the cells were stained with FITC-conjugated anti-CD8α mAb (eBioscience) for 30 min at 4 ℃, washed with PBS, fixed with 4% paraformaldehyde, and permeabilised with PBS containing 0.5% saponin (both from BD, Shanghai, China). Cells were incubated with PE-labelled anti-INF-γ McAb (eBioscience) for 30 min at 4 ℃, washed with PBS, and analyzed by flow cytometry.

Hepatitis B virus core antigen-specific cytotoxic T lymphocytes activity

The former stimulated splenocytes (5×10^6/mL) were used as effectors, and the P815/c cell line was used as target cells. P815/c cells were seeded at a density of 5×10^4 cells/well in 96-well plates. Effector cells were incubated with P815/c at different effector and target (E/T) ratios (12.5:1, 25:1 or 50:1) at 37 ℃ under 5% CO_2 for 4 h. The HBcAg-specific CTL activity was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison WI, United States) for lactate dehydrogenase (LDH) release according to the manufacturer's instructions. The absorbance values of the supernatants were recorded at optical density 450 nm. Percent cytotoxicity was calculated as follows: [(Experimental release - Effector spontaneous release - Target spontaneous release)/(Target maximum release - Target spontaneous release)] × 100%.

Statistical analysis

Results were expressed as mean ± SD. Differences between groups was determined using Student's *t* test, and the differences between two or more groups were determined using a one-factor analysis of variance. Data were considered statistically significant at $P < 0.05$.

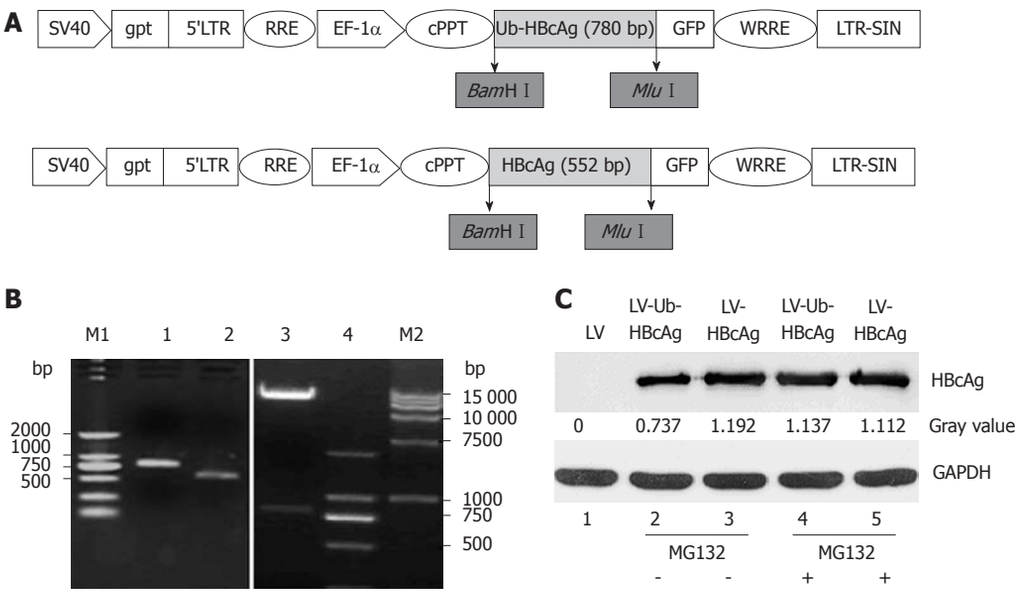

Figure 1 Schematic diagram, electrophoresis of ubiquitinated hepatitis B virus core antigen and *HBcAg* genes, pWPXLd-Ub-HBcAg digested by *Bam*H I and *Mlu* I, and HBcAg protein expression (about 21 kDa). A: Schematic diagram of pWPXLd vector; B: Lane 1, ubiquitinated hepatitis B virus core antigen (Ub-HBcAg) polymerase chain reaction (PCR) product (780 bp); lane 2, HBcAg PCR product (552 bp); lane 3, The digested products pWPXLd-Ub-HBcAg by *Bam*H I and *Mlu* I; lane 4 and lane M1, DNA marker 2000; lane M2, DNA marker 15 000; C: 293T cells were transduced with lentiviral vector (LV), LV-Ub-HBcAg or LV-HBcAg and cultured for 48 h. MG-132 (10 mmol) was added for 24 h before harvesting the cells. Cell lysates (10 mg) were analyzed by immunoblotting with an anti-HBc antibody. Relative expression of HBcAg was calculated by a gray value.

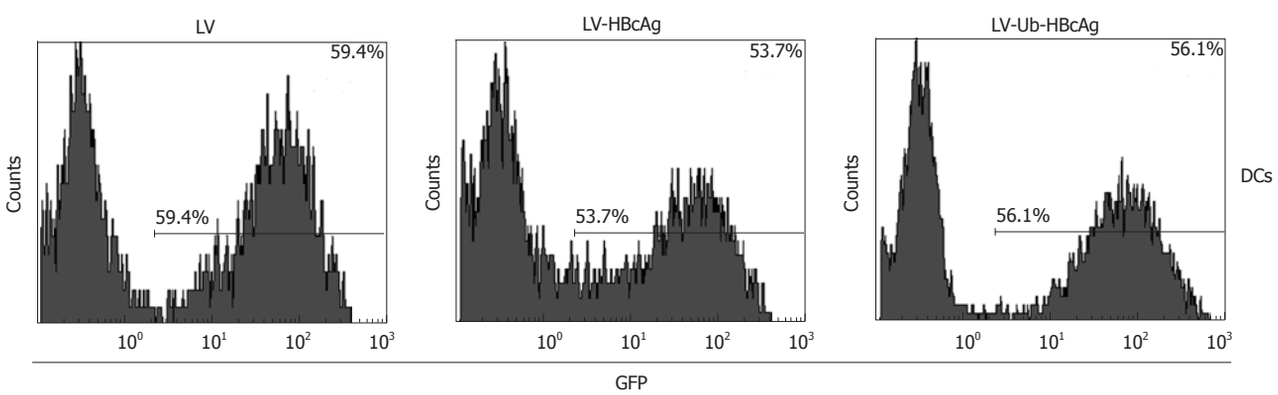

Figure 2 Transduction of dendritic cells with lentiviral vectors expressing green fluorescent protein was evaluated by flow cytometry. Dendritic cells (DCs) were seeded in six-well plates at 1 × 10^6 cells/well. Lentiviral vectors ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg), lentiviral vectors hepatitis B virus core antigen (LV-HBcAg) or lentiviral vector (LV) was added at an multiplicity of infection of 20. GFP: Green fluorescent protein.

RESULTS

Construction of lentiviral vectors vectors and transduced dendritic cells

A 780 bp fragment of the *Ub-HBcAg* gene was cloned into pWPXLd (Figure 1B) and packed into LVs. The *HBcAg* gene was similarly assembled as a control. After concentration, all vectors in the study achieved a titration of approximately 7.5 × 10^8 transducing units/mL. The construction procedure is shown in Figure 1A. As expected, Ub-HBcAg expression was lower than that of HBcAg and recovered to the same level as that of HBcAg when MG-132 was added to the culture (Figure 1C). The transduction efficiency of LVs into DCs was evaluated using flow cytometry by detecting GFP expression (Figure 2). On day 4 after infection, 56.1% of GFP-expressing DCs were detected.

Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen-induced dendritic cell maturation increased IL-12 production and enhanced lymphocyte proliferation

At the end of the treatment, the amount of DCs ($CD11c^+$) was 75% by fluorescence-activated cell sorting analysis. MHC Ⅱ, CD80 and CD86 molecules, which are characteristic of DCs, were used to evaluate DC differentiation and maturation. These molecules were highly expressed in DCs transduced with LV-Ub-HBcAg compared with those transduced with the alternatives (Figure 3). DC function was evaluated by IL-12 secretion and promotion of lymphocyte proliferation. DCs transduced with LV-

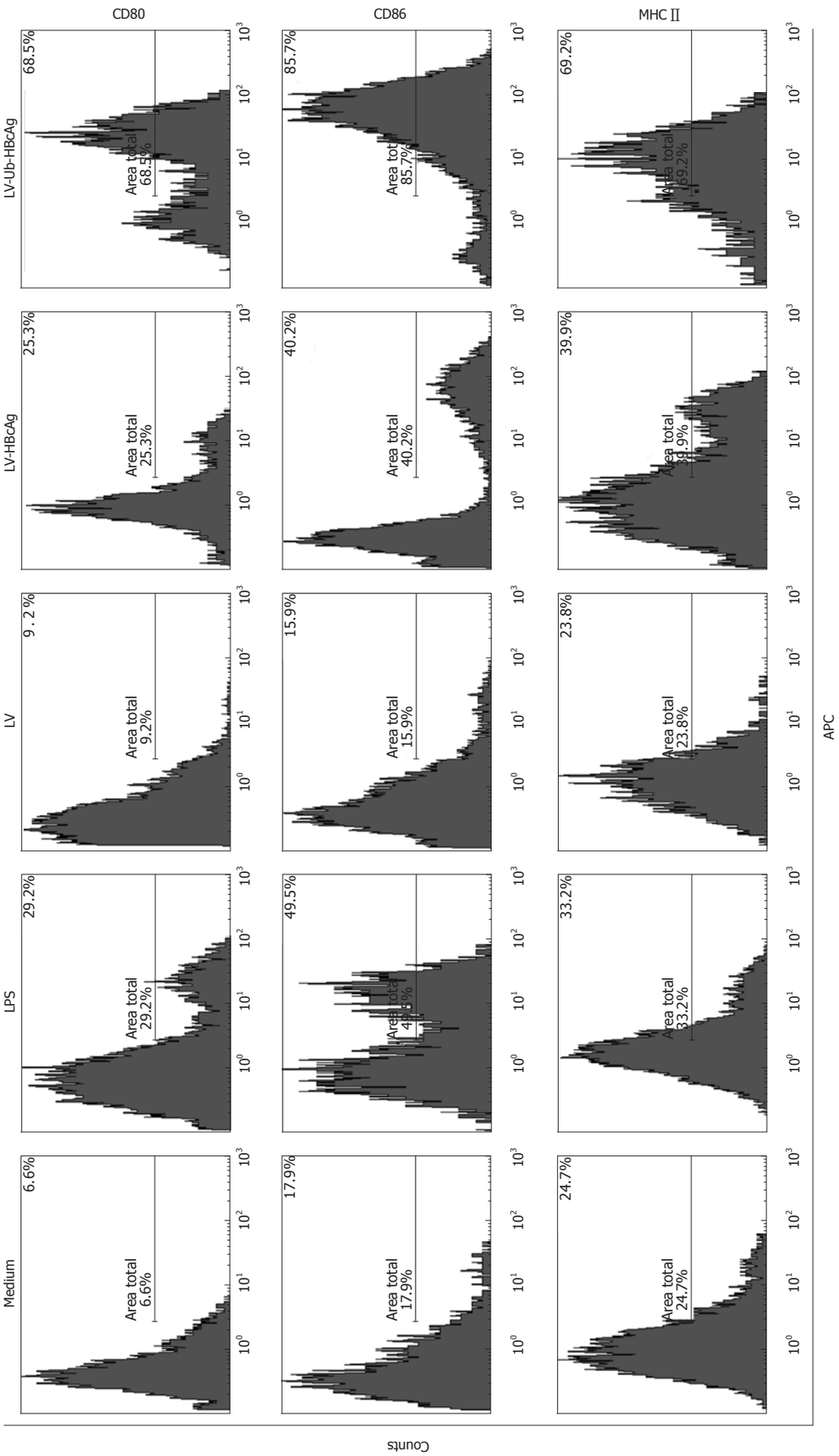

Figure 3 Percentage of dendritic cell surface molecules. In the lentiviral vectors ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg) group, the expression of major histocompatibility complex (MHC) class Ⅱ, CD80 and CD86 molecules characteristic of dendritic cells (DCs) was significantly higher than that in the lipopolysaccharide (LPS) or lentiviral vectors hepatitis B virus core antigen (LV-HBcAg) group. The results represent one of three experiments. LV: Lentiviral vector; APC: Antigen-presenting cell.

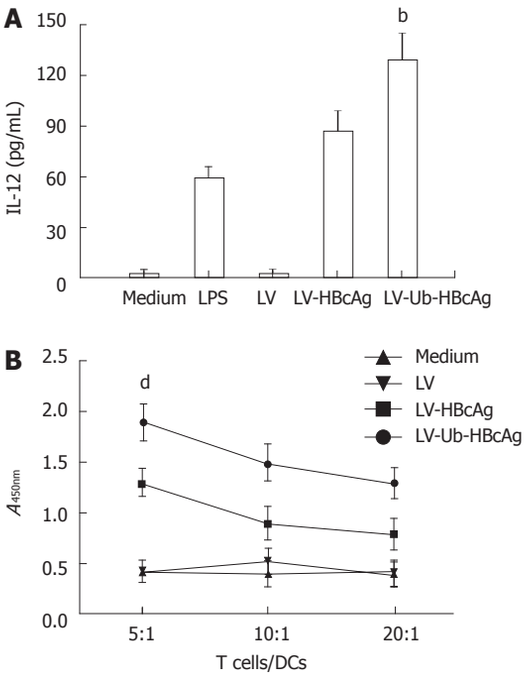

Figure 4 Interleukin-12 secretion of dendritic cells, and detection of the T lymphocyte proliferation response. A: Interleukin-12 (IL-12) production was measured by enzyme-linked immunosorbent assay. Experiments were repeated in triplicate with similar results. Data shown represent the mean ± SD. $^{b}P < 0.01$ *vs* lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group; B: T cell proliferation ability. All experiments were performed twice under the same conditions. $^{d}P < 0.01$ *vs* LV-HBcAg group. DC: Dendritic cell; LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector.

Ub-HBcAg showed significantly higher levels of IL-12 production and lymphocyte proliferation than did the others ($P < 0.01$) (Figure 4A and B). Lymphocyte proliferation capacity was enhanced by the T cell/DC ratio.

Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen boosted cytokine production and CD8$^+$ T cells elicited from proliferative T cells in vitro

T cells stimulated by DCs transduced with LV-Ub-HBcAg showed increased IFN-γ and IL-2 secretion compared with DCs transduced with LV-HBcAg (Figure 5). No significant difference between the two groups was observed for IL-4 and IL-10 production. CTLs were analyzed by intercellular IFN-γ and CD8α$^+$ levels. The levels and intensities of IFN-γ expression were higher in the LV-Ub-HBcAg than in the LV-HBcAg samples (Figure 6A and B), suggesting that DCs transduced with LV-Ub-HBcAg were effective for inducing CTLs.

Enhancement of cytotoxic T lymphocyte activity in dendritic cells transduced with Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen

The LDH relaxation index was determined to evaluate the specific cytotoxicity of T lymphocytes in response to different LV-transduced DCs. HBcAg-specific CTL activities with different effector/target ratios are shown in Figure 7. T lymphocytes from the LV-Ub-HBcAg-

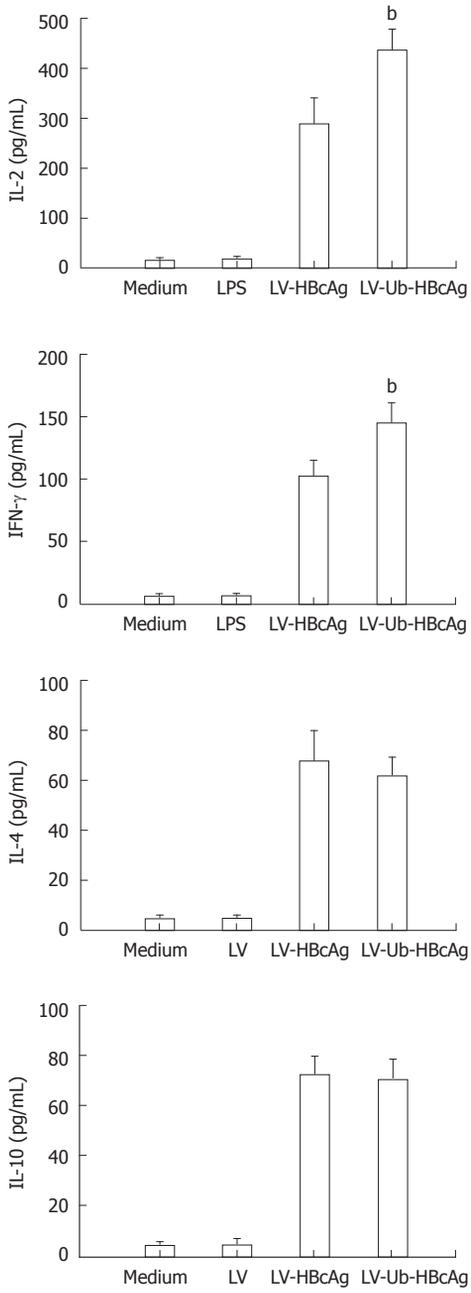

Figure 5 Cytokine production. Cytokine secretion of proliferative T cells. Data represent the mean ± SD. $^{b}P < 0.01$ *vs* lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group. LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector; INF-γ: Interferon-γ; IL: Interleukin.

transduced DCs killed 55.0% ± 4.3% target cells at an effector and target ratio of 50:1, which was significantly higher than that of the LV-HBcAg-transduced DC group (32.4% ± 5.2%) ($P < 0.01$). Accordingly, these results indicated that LV-Ub-HBcAg-transduced DCs induced strong specific CTL responses.

DISCUSSION

The development of novel immunotherapies has been highly anticipated because HBV infection is one of the

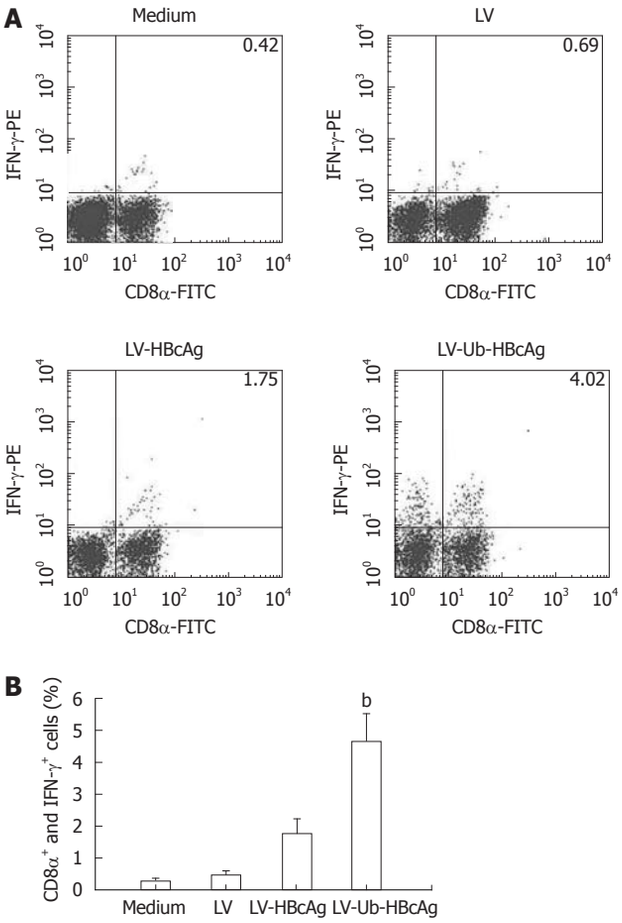

Figure 6 Intracellular cytokine analysis. A: The proliferative T cells were suspended in complete RPMI 1640 (2 × 10^6/mL), Intracellular cytokine analysis by flow cytometry using CD8α-FITC and Interferon (IFN)-γ-PE antibodies. The results are representative of one of three experiments; B: The presence of $CD8\alpha^+$ and IFN-γ^+ cells . $^bP < 0.01$ *vs* lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group. LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV-HBcAg: Lentiviral vector-encoding hepatitis B virus core antigen; LV: Lentiviral vector.

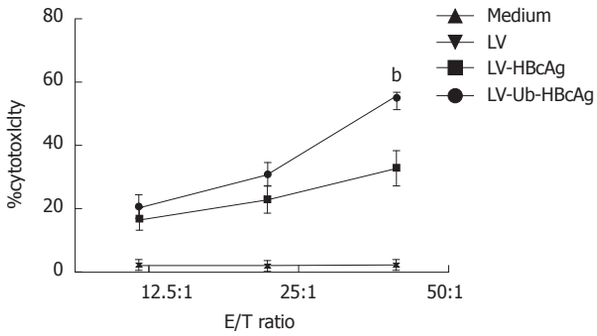

Figure 7 Cytotoxic response of proliferative T cells. Effector cells were incubated with P815/c at different effector/target (E/T) ratios (12.5:1, 25:1 or 50:1) for 4h. Experiments were repeated three times with similar results. Data represent the mean ± SD. $^bP < 0.01$ *vs* lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group. LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector.

leading causes of cancer or hepatocellular carcinoma-related death. Several studies have demonstrated that the main cause of viral persistence during HBV infection is an inadequate antiviral immune response to the viral antigens[13,14]. The viral-specific $CD8^+$ T cell response plays an important role in the process of viral clearance. Patients with chronic hepatitis B (CHB) or therapeutic failure show deficient Th1 immunity associated with inefficient $CD8^+$ T cell cytotoxicity[15]. Therefore, induction of CTL responses specific to HBV represents a promising strategy to protect against HBV infection.

DCs are key antigen-presenting cells that induce primary and memory immune responses. Impaired DC function is found in chronic HBV infection, in which patients are generally in an immunocompromised state of immune tolerance[16-18]. Considerable effort has been made to introduce antigens into DCs in the forms of peptides, proteins, or transgenic protein antigens using viral vectors[19,20]. Various viral vectors, including poxvirus and adenovirus, have been used to genetically modify DCs, but low transduction efficiencies have limited their application. We chose LVs as a gene transfer vector because they can transduce non-dividing, monocyte-derived DCs and bone marrow-derived DCs with very high transduction efficiencies[21,22]. Several reports have demonstrated that immunizing mice with LVs by delivering viral or tumor model antigens mice elicited broad and long-lasting specific immune responses. For example, lentiviral transduction of DCs expressing ovalbumin effectively processed and presented the ovalbumin antigens and induced ovalbumin-specific T cell responses[23]. Our results confirmed that lentivirus-mediated gene transfer could offer the unique opportunity to investigate the biologic activity of DCs.

The ubiquitin-proteasome system (UPS) is a highly selective adenosine-5'-triphosphate-dependent proteolytic system present in all eukaryotic cells and plays a key role in antigen presentation[8]. It is well established that short antigenic peptides must be presented on MHC class I molecules of target cells to be recognized by specific CTLs. Proteasomes are responsible for the proteolysis of intracellular proteins, including viral antigens, to generate MHC class I ligands.

Attachment of Ub to a protein is the initial signal for targeted protein degradation. To prevent fusion gene (*Ub-HBcAg*) cleavage by deubiquitination enzymes, we constructed a pWPXLd vector encoding HBcAg fused with Ub, in which the Ub C-terminal glycine was replaced with alanine[24]. Additionally, HBcAg with a modified N-terminal Met residue was replaced by Arg. By this method, the fusion protein can be quickly recognized by the UPS, resulting in a promotion of HBcAg degradation[25-27]. In our study, Western blotting analyses identified efficient expression of HBcAg from the 293T cells transduced with recombinant LVs. Ub-fused HBcAg was converted into an excellent substrate for the UPS. We found that the 293T cells transduced with LV-Ub-HBcAg showed low levels of protein expression in the absence of MG-132.

Immature DCs expressed low levels of surface MHC molecules, producing almost no expression of CD40, CD80 or CD86. Fully matured DCs showed strong surface expression of MHC class Ⅱ and co-stimulatory mo-

lecules (CD80 and CD86). In our study, the surface molecules CD80, CD86, and MHC class Ⅱ DCs were markedly upregulated by LV-Ub-HBcAg stimulation, whereas no significant change was observed after LPS or LV-HBcAg stimulation. An important sign of mature DCs is IL-12 secretion. Mature DCs secrete high levels of IL-12 that promote activation of effector cells (e.g., natural killer cells, lymphokine-activated killer cells, tumor-infiltrating lymphocytes and macrophages) and induce a variety of cytokines (e.g., IFN-γ, GM-CSF, IL-2 and IL-8)[28]. IL-12 is produced by mature DCs in response to infection by various intracellular pathogens. This response plays a critical role initiating a specific T cell-mediated immune response and drives Th1 cell activation and differentiation[29,30]. We examined IL-12 production after adding different maturation factors to the culture medium. As expected, IL-12-induced LV-Ub-HBcAg production of DCs was markedly elevated compared with that produced by other treatments. In this study, LV-Ub-HBcAg-transduced DCs not only promoted DC surface molecule expression, but also promoted further secretion of IL-12, which helped stimulate the immune response.

Higher rates of intracellular antigen traffic should increase the number and varieties of peptides available for MHC class Ⅰ binding, which may result in an increase in the cell immune response to the expressed antigen. DCs pulsed with HBV antigens effectively abrogated CTL tolerance in HBV transgenic mice. Chen *et al*[4] demonstrated that DCs loaded with HBcAg not only induce the production of HBV-specific T cells but also restore the impaired function of such cells. The DCs generated by transfection of LV-Ub-HBcAg were able to stimulate proliferation of naive allogeneic T lymphocytes and to increase the number of antigen-specific $CD8^+$/IFN-γ^+ T cells *in vitro*. Th1 cells primarily secrete IL-2 and IFN-γ, whereas Th2 cells secrete type Ⅱ cytokines IL-4 and IL-10. Th1/Th2 immune balance plays a key role in the outcome of HBV infection. Dominant Thl cells tend to lead to an acute self-limited HBV infection; dominant Th2 cells tend to occur with a chronic persistent HBV infection. In our study, we observed that the LV-Ub-HBcAg group had higher levels of both IL-2 and IFN-γ in the lymphocyte supernatant compared with those in the LV-HBcAg group. This was further supported by enhanced levels of IFN-γ-producing $CD8^+$ T cells. These results clearly indicate that the immune responses were directed toward a Th1 type rather than a Th2 type. Th1 cells are correlated with the induction of CTL activity, which is beneficial for viral or tumor eradication[31,32]. In this study, the LV-Ub-HBcAg-transfected DCs stimulated T lymphocytes and generated antigen-specific cytotoxic T lymphocytes more efficiently than those of the LV-HBcAg-transfected DCs. Thus, LVs carrying Ub-fused HBcAg effectively activated antigen-specific $CD8^+$ T cells. Inadequate endogenous antigen presentation by MHC class Ⅰ molecules to $CD8^+$ T cells is one of the reasons for the failure of the immune system to eliminate pathogens. Patients with CHB or therapeutic failure showed deficient Th1 immunity associated with inefficient $CD8^+$ T cell cytotoxicity. In our study, enhanced antigen presentation increased the number of antigen-specific $CD8^+$/IFN-γ^+ T cells in the LV-Ub-HBcAg-transfected DC group. Ub-fused HBcAg was rapidly degraded by the proteasome, resulting in efficient production of a variety of peptides, including many CTL epitopes that may be presented by many types of MHC class Ⅰ molecules.

In summary, we have successfully transfected murine bone marrow-derived DCs with LVs encoding the Ub-HBcAg fusion gene. The Ub-HBcAg-transfected DCs proliferated and generated HBcAg-specific CTLs more efficiently than did the HBcAg-transfected DCs. Therefore, this novel strategy may have therapeutic value that can be applied to the treatment of infectious diseases.

COMMENTS

Background

Hepatitis B virus (HBV) infection is a serious public health problem. Defective cytotoxic T lymphocytes (CTLs) may lead to persistent HBV infection, and the defective CTL response was ascribed to the impaired dendritic cell (DC) function. Promoting and improving DC function is a promising approach to combating persistent HBV infection. Ubiquitin (Ub) is a highly conserved small regulatory protein, and the Ub-mediated processing of antigens is rapid and efficient and stimulates cell-mediated immune responses.

Research frontiers

Ub-fused melanoma antigens could induce antigen proteins to execute proteasome-dependent degradation and created epitopes of major histocompatibility complex (MHC) class Ⅰ, resulting in the preferential activation of antigen-specific $CD8^+$ T cells.

Innovations and breakthroughs

The study reported for the first time the capacity of DCs transfected with lentivirus encoding the ubiquitinated hepatitis B virus core antigen (Ub-HBcAg) to stimulate lymphocyte proliferation and to generate antigen-specific CTLs.

Applications

The authors found that lentivirus encoding Ub-HBcAg effectively could induce DC maturation. The mature DCs efficiently induced T cell polarization to Th1 and generated HBcAg-specific CTLs. These results may be helpful in seeking novel approaches to the control of persistent HBV infection.

Terminology

Ubiquitin is a highly conserved small regulatory protein, ubiquitous in eukaryotes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes.

Peer review

The topic is novel, with very few articles published in this field till now. The manuscript is well organized with objectives, methods, results being adequately described, and the conclusions are based on the results found. Tables and figures are appropriate. Statistical analysis needs better description by providing *P* values through the text where comparisons between groups are present.

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ORIGINAL ARTICLE

Loss of Wnt5a and Ror2 protein in hepatocellular carcinoma associated with poor prognosis

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Abstract

AIM: To investigate the expression and clinical significance of Wnt member 5a (Wnt5a) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) in hepatocellular carcinoma (HCC).

METHODS: HCC tissues obtained from 85 patients were examined the mRNA expression of Ror2 by real-time reverse transcription polymerase chain reaction and the protein expressions of Wnt5a, Ror2, β-catenin and Ki-67 *via* immunohistochemical staining. The correlation between protein expression and HCC patients' clinicopathology data and prognoses were analyzed.

RESULTS: Compared to nontumorous (hepatitis or cirrhotic) tissues, Ror2 mRNA expression was clearly decreased in HCC. Ror2 and Wnt5a protein expressions in the majority of HCC patients (63% and 77%, respectively) was significantly less in tumor tissues, as compared to adjacent nontumorous tissues, and this reduction was correlated with increasing serum α-fetoprotein and tumor stage. In 68% (58/85) of the HCC cases, the expression of β-catenin in tumor tissues was either downregulated in the cellular membrane, upregulated in the cytoplasm, or both. Survival analysis indicated that Wnt5a and Ror2 protein expressions could be regarded as independent prognostic factors for HCC; HCC patients with decreased Wnt5a or Ror2 protein expression had a poorer prognosis than those with elevated Wnt5a and Ror2 expression (P = 0.016, P = 0.007, respectively).

CONCLUSION: Wnt5a and Ror2 may serve as tumor suppressor genes in the development of HCC, and may serve as clinicopathologic biomarkers for prognosis in HCC patients.

Key words: Hepatocellular carcinoma; Wnt5a; Receptor tyrosine kinase-like orphan receptor 2; β-catenin; Prognosis

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Geng M, Cao YC, Chen YJ, Jiang H, Bi LQ, Liu XH. Loss of Wnt5a and Ror2 protein in hepatocellular carcinoma associated with poor prognosis. *World J Gastroenterol* 2012; 18(12): 1328-1338 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i12/1328.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i12.1328

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently occurring tumors worldwide. It develops mostly in cirrhotic livers, and risk factors include chronic infection by the hepatitis B and C viruses (HBV and HCV), as well as nonviral liver diseases[1,2]. Unfortunately, the cellular mechanisms of hepatocarcinogenesis remain poorly understood. Recent advances have shown that apart from autocrine stimulation by growth factors such as insulin-like growth factor-Ⅱ and transforming growth factor-α, the dysregulation of at least four different growth regulatory pathways is frequently involved in hepatocarcinogenesis[3,4]. These signaling pathways include the retinoblastoma, the transforming growth factor-β, the tumor protein 53, and the wingless-type murine-mammary-tumor virus integration site family (Wnt). These pathways also interfere with each other at different levels[2,5,6].

The Wnt family of genes encodes a large and diverse group of signaling molecules involved in the patterning, proliferation, and differentiation of a variety of organs and cell types[7,8]. The Wnt ligand binds to its receptor Frizzled and the low-density lipoprotein receptor-related proteins (Lrp) 5 and 6 to activate the canonical Wnt/β-catenin signaling pathway, or functions through β-catenin-independent (noncanonical) pathways which include the planar cell polarity and Wnt/Ca^{2+} pathways[9]. Wnt ligands are typically classified into canonical and non-canonical Wnts by the pathways they work through[9-11].

The Wnt member 5a (Wnt5a) is one of the most highly investigated non-canonical Wnts and has been implicated in almost all aspects of non-canonical Wnt signaling[12-14]. In terms of cancer developmental research, Wnt5a has lived in the shadow of its better-characterized relatives. This was largely because of its apparent inability to transform cells or signal through the canonical β-catenin pathway that is so important in cancer[15-18]. Recent work with a wide of human tumors has indicated that Wnt5a has a critical role in malignant progression, but there is conflicting evidence as to whether that role is tumor-promoting or tumor-suppressing[17-22]. We have shown that Wnt5a has a tumor suppressing effect in HCC and is probably associated with HBV infection[23,24]. Emerging evidence suggests that the functions of Wnt5a can be drastically altered depending on the availability of key receptors[17,18,25]. It was recently reported that an alternative Wnt receptor, receptor tyrosine kinase-like orphan receptor 2 (Ror2), an orphan tyrosine kinase, mediates Wnt5a-initiated noncanonical signaling and is required for the Wnt5a-mediated inhibition of canonical signaling[25,26].

The Ror2 receptor belongs to the receptor tyrosine kinase superfamily[25]. This large protein family is involved in regulating diverse cellular processes such as the cell cycle, cell migration, proliferation and differentiation[27]. In addition, the Ror2 protein and its homolog Ror1 play essential roles during development. Mutations of the Ror2 receptor, resulting in protein misfolding or premature truncation, have been associated with human diseases such as dominant Brachydactyly type B and recessive Robinow syndrome[28]. Currently, investigations to elucidate the role of Ror2 in cancer have shown paradoxical results, indicating that Ror2 was overexpressed in oral and renal cell cancer and metastatic melanoma, but downregulated in colon cancer[29-31]. These different effects appear to be dependent on the cancer type and signaling pathway[32].

Here, we investigate the expression and clinical significance of Ror2, Wnt5a and β-catenin in HCC.

MATERIALS AND METHODS

Patients and specimens

We collected tumors from 85 consecutive patients who had undergone surgery for HCC at the Jinan Military General Hospital from January 2006 to September 2010. The Ethics Committee of the Jinan Military General Hospital approved the protocol of this study. Among the 85 patients, 55 had serum α-fetoprotein (AFP) ⩾ 30 μg/L, and 73 were sera positive for hepatitis B surface antigen (HBsAg). On gross examination, 3 cases had tumor sizes that were ⩽ 2 cm, and 82 had tumor sizes > 2 cm (median tumor size, 6.1 cm; range, 1.0-16 cm). Histopathological diagnoses were made according to the pathological classification system of the World Health Organization (2000), and the tumor was staged following the tumor-node-metastasis classification of the International Union Against Cancer[33]. Nine cases were well differentiated; 60 cases were moderately differentiated; and 16 cases were poorly differentiated. In total, 56 HCC cases had liver cirrhosis; 25 cases had chronic hepatitis; and 4 cases had basically normal liver tissue. We also collected 3 cases of lung metastasis. Furthermore, tissues of comparative normal liver obtained during surgery for liver cholelithiasis (n = 3) and HBV-infected liver biopsies (n = 5) were studied.

Nineteen of the 85 cases included chronic (n = 8) and cirrhotic (n = 11) HCC. From these, fresh tissues were obtained immediately after resection, including HCC tumor and adjacent nontumorous liver tissues. In addition, normal liver tissues (n = 3) with no HBV infection were obtained during surgery for liver cholelithiasis. In these 22 cases, one portion of the fresh tissue was snap frozen in liquid nitrogen immediately and stored at -80 ℃; the remainder portion was fixed in 10% buffered formalin and embedded in paraffin.

The available patient clinicopathological information included gender, age, serum AFP, serum HBsAg, tumor size, tumor stage, histological grade and cancer-specific survival time.

Extraction of RNA and real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from 10-mm frozen HCC tissue sections. To isolate the RNA from defined areas containing ⩾ 80% tumor cells, all tumors were manually microdissected under direct visual control through a dis-

secting microscope. Total RNA in the frozen tissues was extracted using Trizol (Invitrogen) following the manufacturer's recommendations. Total RNA was digested with DNase I (Invitrogen) and was used for the first-strand cDNA reaction. The reaction mixture consisted of 5 μg of DNase I-treated RNA, 1 × reverse transcriptase buffer, 2.5 mmol dNTP mix, 3.5 μmol oligo primer, and 2.5 U/mL MultiScribe™ reverse transcriptase (PE Applied Biosystems). Each sample was handled using the same protocol, with the exception that reverse transcriptase was added to exclude the presence of interference from genomic DNA.

Real-time reverse transcription polymerase chain reaction (qRT-PCR) was carried out using SYBER green dye in a Rotor Gene 3000 Detection System (Corbett Research, Sydney, Australia). Each SYBER green reaction (25 μL) contained one microliter diluted cDNA and 10.5 μL SYBR Green PCR Master Mix, as well as 5 pmol forward and reverse primer (Ror2: forward 5'-AGGTGCCTATGCAAGTTCA-3', reverse 5'-TGTGCGAGGTTTAAGGTCTA-3'). Samples were activated by incubation at 95 ℃ for 5 min and denatured at 95 ℃ for 20 s. This was followed by annealing at 60 ℃ for 20 s and extension at 72 ℃ for 20 s, for 40 cycles. In all of the cDNA samples, gene expressions of Ror2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse 5'-GAAGATGGTGATGGGATTTC-3'), an internal quantitative control, were determined by SYBR green fluorescence using the Rotor-Gene 3000; the ratios of Ror2 to the housekeeping gene *GAPDH* represented the normalized relative levels of Ror2 expression. A non- template negative control was also included in each experiment. Analyses of all tumor samples were carried out at least twice, and the mean value was calculated.

Immunohistochemistry

Immunohistochemical staining was performed on thin sections (4 μm) of paraffin-embedded archival tissue. The samples were dewaxed with xylene/ethanol before antigen retrieval (i.e., pressure cooked for one minute at full pressure, 15 psi, in 0.001 mol/L EDTA buffer, pH 8.0). The primary antibodies used were: Wnt5a (LS-C47384, Lifespan, 1:200), Ror2 (PAB3386, Abnova, 1:200), β-catenin (C19220, BD Transduction Laboratories, 1:400) and Ki-67 (MIB-1, Dako, 1:100). Immunohistochemical staining of antibodies was done using the Dako Envision Plus System (K5007, Dako). The antibody binding was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) before brief counterstaining with Mayer's hematoxylin. For monoclonal antibodies of mouse origin, negative controls were obtained using isotypic mouse immunoglobulin in the same dilution as the primary antibody of concern. All control experiments gave negative results.

Evaluation of immunostaining

Two authors (Cao YC and Jiang H) who had no knowledge of the patients' clinical status reviewed all of the immunostained sections. Cases with discrepant results were re-evaluated jointly until agreement was reached. For expression of Wnt5a, Ror2 and β-catenin protein, in cases with multiple areas of low intensity that occurred during evaluation of immunostaining, five areas were selected at random and scored.

The degree of immunohistochemical staining was recorded using a semi- quantitative and subjective grading system that considered both the intensity of staining and the proportion of tumor cells that had an unequivocal positive reaction. Grades for stain intensity were: 0: No staining; 1: Weak staining; 2: Positive staining; and 3: Strong staining. For rating stained areas: 0: No staining; 1: Positive staining in < 10% of tumor cells; 2: Positive staining in 10% to 50% of tumor cells; 3: Positive staining in > 50% of tumor cells. The staining index was calculated as the staining intensity multiplied by the positive area.

Ki-67-positive cells were counted by viewing ≥ 200 HCC cells from ≥ 10 randomly selected fields. The percentage of antigen-positive nuclei among the total number of nuclei counted was calculated to obtain the nuclear labeling index (LI).

In the subsequent statistical analysis, the cutoff points for the staining index categories were mainly based on median values, as well as each marker's frequency distribution curve and the size of the subgroups. Therefore, cytoplasmic Wnt5a and Ror2 and membranous β-catenin staining indices were categorized by their median value as high (> 4) or low (0-4), and the cytoplasmic β-catenin staining index was categorized as high (> 3) or low (0-3). However, nuclear β-catenin expression was categorized based on the absence (staining index = 0) or presence (staining index ≥ 1) of staining. The Ki-67 labeling indices were divided into two groups (LI < 10% and LI ≥ 10%).

Follow-up and statistical analysis

To determine the prognostic factor, the outcome of the 82 patients was determined by reviewing their medical charts. The follow-up period ranged from one to 54 mo (average: 31.3 mo; median: 27.0 mo). The end point in the analysis was HCC-related death. The overall and disease-free survival rates were estimated using the Kaplan-Meier method and compared with the log-rank test. The prognostic analysis was carried out with univariate and multivariate Cox regressions models.

The differences in Ror2 mRNA expression between HCC and nontumorous liver tissue was statistically analyzed using Student's *t*-test and one-way analysis of variance (ANOVA) for multiple comparisons. The correlations between the clinicopathological parameters and Wnt5a, Ror2 and β-catenin protein expression were analyzed using the χ^2 or Fisher's exact tests.

Pearson's correlation was used to determine the correlation between mRNA and protein expression, as well as between the expressions of different proteins. All sta-

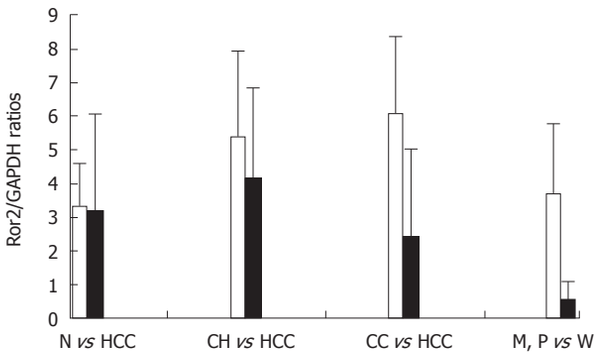

Figure 1 Real-time reverse transcription-polymerase chain reaction analysis of *Ror2* gene (mRNA) expression in hepatocellular carcinoma, chronic hepatitis, cirrhotic and normal liver tissue. Ror2: Receptor tyrosine kinase-like orphan receptor 2; HCC: Hepatocellular carcinoma; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Bars: mean; Columns: SD; N: Normal; CH: Chronic hepatitis; CC: Cirrhosis; M, P, W: Moderately, poorly, and well differentiated tumor tissues, respectively.

tistical calculations were carried out using SPSS software (for Windows, version 13.0). A significant difference was defined at $P < 0.05$.

RESULTS

Ror2 gene and protein expression in hepatocellular carcinoma

The *Ror2* gene (mRNA) expression levels relative to that of GAPHD in normal, HCC, chronic hepatitis, cirrhotic liver and adjacent nontumorous liver tissues are shown in Figure 1. Ror2 mRNA levels were elevated in chronic hepatitis (5.420 ± 5.492, $n = 11$) and cirrhotic liver tissues (6.128 ± 5.252, $n = 8$) compared to that of normal (3.381 ± 1.182, $n = 3$) and HCC (3.189 ± 3.856, $n = 19$). Based on Student's *t*-test, statistically significant differences were found between the Ror2 mRNA levels in HCC *vs* adjacent nontumorous (chronic hepatitis or cirrhotic) liver tissues ($P = 0.029$), but not between HCC and normal liver tissues ($P = 0.934$) or normal and adjacent nontumorous liver tissues ($P = 0.094$). The Ror2 mRNA level in moderately and poorly differentiated tumor tissues ($n = 16$) was greater by 7.2-fold ($P = 0.014$) than the level in well-differentiated tumor tissues ($n = 3$). No significant differences were found between *Ror2* gene expression levels and other clinicopathological findings such as age, serum AFP concentration, tumor size, and HCC tumor stage.

Immunohistochemistry was performed to evaluate Ror2 protein expression in tumor and non-tumorous liver cells. In non-tumorous liver cells and HCC tumor cells, Ror2 protein expression was displayed in the cytoplasm, but in stromal cells Ror2 protein was not observed. In comparative normal liver cells Ror2 was negative or weakly expressed (Figure 2A and B), whereas all chronic hepatitis, cirrhotic, and dysplastic liver cells exhibited positive immunostaining for Ror2 (Figure 2C and D), In 62/85 (72.9%) of the HCCs, Ror2 immunostaining was reduced or absent (Figure 2E and F).

Table 1 The relationship between receptor 2 expression and clinicopathological features in hepatocellular carcinomas

Variables	*n*	Ror2 immunoreactivity		*P*
		Low	High	
Gender				0.890
Male	77	56	21	
Female	8	6	2	
Age (yr)				0.725
< 53 (median)	38	27	11	
⩾ 53	47	35	12	
Serum AFP level (μg/L)				< 0.001
< 30	30	14	16	
⩾ 30	55	48	7	
HBsAg				0.862
Positive	73	53	20	
Negative	12	9	3	
Tumor size (cm)				0.116
⩽ 2	3	1	2	
> 2	82	61	21	
Histological grade				0.090
Well differentiated	9	7	2	
Moderately differentiated	60	40	20	
Poorly differentiated	16	15	1	
Liver cirrhosis				0.553
Present	56	42	14	
Absent	29	20	9	
T classification				< 0.001
T1	3	1	2	
T2	30	14	16	
T3	40	36	4	
T4	12	11	1	
Total	85	62	23	

AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; Ror2: Receptor tyrosine kinase-like orphan receptor 2.

A significant correlation was found between the normalized *Ror2* gene expression ratio and the protein expression level in normal, tumor and non-tumorous liver tissues ($r = 0.254$, $P = 0.021$). Furthermore, statistical comparisons between Ror2 mRNA expression and patients' clinicopathological features revealed a significant negative association between Ror2 mRNA and tumor stage ($P < 0.001$), and between Ror2 mRNA and serum AFP ($P < 0.001$). However, there were no significant differences between Ror2 protein expression and the other clinicopathological findings in HCC (Table 1).

Wnt5a protein expression in hepatocellular carcinoma

Wnt5a protein expression was observed in the cytoplasm of non-tumorous liver and tumor cells, but nowhere in stromal cells. There was little or no Wnt5a seen in normal liver cells. However, all chronic hepatitis, cirrhosis and dysplastic liver cells exhibited strong positive immunostaining for Wnt5a. In contrast, in 65/85 (76.5%) of HCC patients, Wnt5a immunostaining was reduced or absent compared to the levels in adjacent nontumorous (hepatitis and cirrhotic) tissues (Figure 3A). There was a significant negative correlation between Wnt5a expression and tumor stage ($P < 0.001$), and between Wnt5a and serum AFP ($P = 0.016$). However, there were no significant

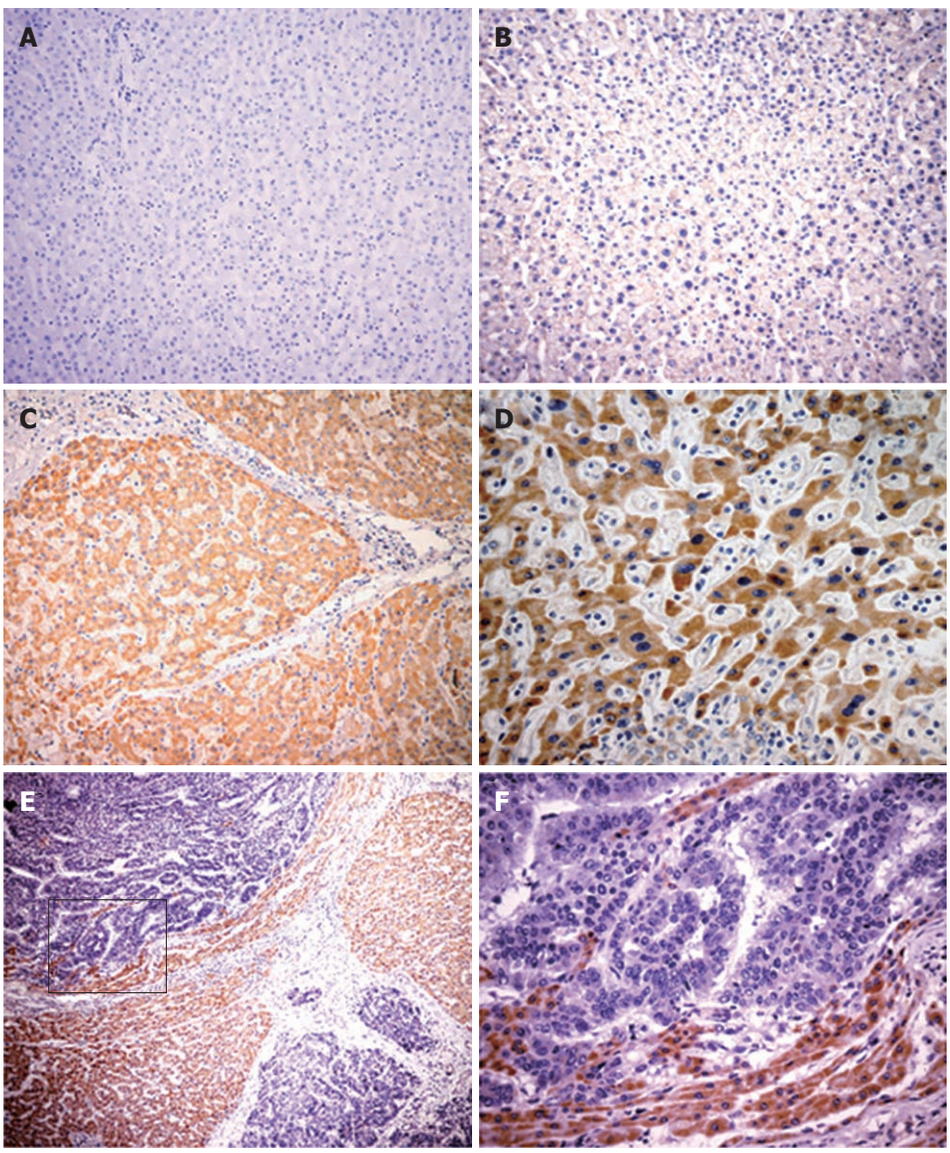

Figure 2 Immunohistochemical staining for Ror2 in hepatocellular carcinoma. Patient-matched normal liver cells showed negative (A) or weak expression (B) of receptor tyrosine kinase-like orphan receptor 2 (Ror2). Liver cirrhosis cells (C) and dysplastic liver cells (D) exhibited strong positive immunostaining for Ror2. Tumor cells (E, F) showing negative Ror2 staining in hepatocytes, while strong cytoplasmic staining is seen in adjacent nontumorous cells. Original magnification, 200 × in A; 400 × in B and D; 100 × in C.

associations between Wnt5a protein expression and the other clinicopathological features of HCC patients.

β-catenin protein expression in hepatocellular carcinoma

In non-neoplastic liver tissue, a thin membranous β-catenin signal delineated the hepatocytes, and strong membranous and pale cytoplasmic staining of bile ductules was observed. As shown in Figure 3B and C, altered expressions of β-catenin were found in 68.2% (58/85) of HCC cases. These alterations included reductions in the cellular membrane, increases in the cytoplasm, or both, and nuclear accumulation (in 7%, 6/85). However, no evidence of altered β-catenin expression was found in cirrhotic nodules or dysplastic liver cells in adjacent noncancerous liver tissue. In tumor tissues, altered β-catenin expression was significantly associated with a worsening histopathological tumor grade ($P = 0.041$) and was not significantly associated with the other clinicopathological parameters.

Correlations among the protein expressions of Wnt5a, Ror2 and *β*-catenin

Associations among the protein expression levels of Wnt5a, Ror2 and β-catenin are shown in Table 2. Low cytoplasmic Wnt5a expression was positively associated with low cytoplasmic Ror2 expression ($r = 0.411$, $P < 0.001$) and abnormal β-catenin expression ($r = 0.254$, $P = 0.019$) in HCC tissue. Similarly, there was a statistically significant correlation between low cytoplasmic Ror2 expression and abnormal β-catenin expression ($r = 0.267$, $P = 0.014$).

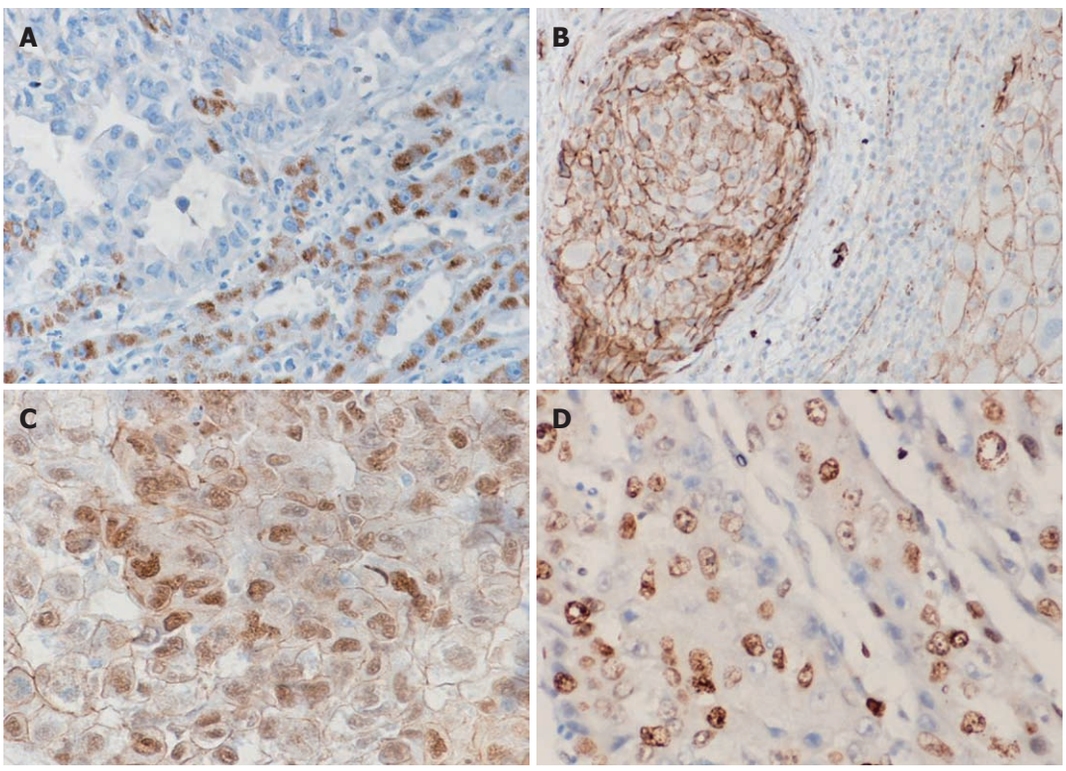

Figure 3 Immunohistochemical staining for Wnt member 5a (A), β-catenin (B, C) and Ki-67 (D) in hepatocellular carcinoma. Original magnification, 400 ×.

Table 2 Correlation between the expression levels of Wnt member 5a, receptor 2 and β-catenin

		Wnt5a			β-catenin		
Variable	*n*	Low	High	*P*-value	*n*	A	*P*-value
Ror2							
Low	62	54	8	< 0.001	15	47	0.014
High	23	11	12		12	11	
β-catenin							
N	27	16	11	0.019			
A	58	48	10				

N: Normal membranous staining; A: Abnormal non-membranous staining; Wnt5a: Wnt member 5a; Ror2: receptor tyrosine kinase-like orphan receptor 2.

Tumor cell proliferation in hepatocellular carcinoma

To investigate the biological functions of proteins in HCC, the Ki-67 LI was assessed in relation to Ror2, Wnt5a and β-catenin status. A strong correlation between a high Ki-67 LI and the reductive loss of Ror2 (r = -0.344, P = 0.002), or Wnt5a (r = -0.278, P = 0.010), but not β-catenin (r = 0.095, P = 0.386) was found (Figure 3D).

Immunohistochemistry for tumor tissues from patients with lung metastasis of hepatocellular carcinoma

A previous study reported that Wnt5a and Ror2 were expressed predominantly in metastatic but not primary lesions of metastatic melanoma, suggesting that Wnt5a and Ror2 might be closely correlated with tumor invasiveness and metastasis[31,34]. To determine whether a similar phenomenon occurs in the metastasis of HCC, three cases of lung metastasis of HCC were included in this study. Immunohistochemical analysis showed that Wnt5a and Ror2 were not expressed in either primary or metastatic lesions (Figure 4A and B), whereas β-catenin- positive staining were detected in the cellular membrane (Figure 4C and D). The Ki-67 LI in tumor tissues was 10%.

Statistical analysis

The median follow-up was 27.0 mo for survivors (range, 1-54 mo). Three patients were lost to follow-up after surgery and were excluded from the survival analyses. The overall survival curve for the remaining 82 HCC cases is shown in Figure 5A. The estimated 1- and 3-year overall rate of survival was 75% and 44%, respectively. Kaplan-Meier analysis was used to compare the survival rates of HCC patients with tumors expressing low or high levels of Wnt5a and Ror2 and normal or abnormal β-catenin (Figure 5B-D).

In a univariate Cox proportional hazard regression model analysis (Table 3), tumor stage (P < 0.001), serum AFP (P = 0.036), and the expressions of Wnt5a (P = 0.024) and Ror2 (P = 0.011) were significantly associated with overall survival. Therefore, patients with tumors having a low expression of Wnt5a and Ror2 had a poorer prognosis than those with tumors of high Wnt5a and Ror2 expression.

Multivariate Cox regression analysis (Table 3), the expression levels of Wnt5a (P = 0.020), and β-catenin (P = 0.013) showed a significant association with overall

Table 3 Univariate Cox and multivariate Cox regression analysis overall survival

Covariate	*P*-value	Risk ratio	95% CI
Univariate			
Sex (male, female)	0.130	0.482	0.187-1.240
Age (< 53 yr, ≥ 53 yr)	0.166	0.640	0.341-1.203
Serum AFP level (< 30 μg/L, ≥ 30 μg/L)	0.036[a]	2.162	1.051-4.449
HBsAg (positive, negative)	0.506	1.621	0.390-6.732
Tumor size (≤ 2 cm, > 2 cm)	0.467	2.089	0.286-15.239
Histological grade (well, moderately, poorly differentiated)	0.268	1.388	0.777-2.482
Liver cirrhosis (present, absent)	0.738	1.123	0.568-6.732
T classification (T1-T4)	< 0.001[a]	2.339	1.487-3.679
Wnt5a (low, high)	0.024[a]	3.288	1.167-9.263
Ror2 (low, high)	0.011[a]	0.323	0.134-0.774
β-catenin (normal, abnormal)	0.052[a]	1.966	0.995-3.885
Ki-67 (mitosis ≤ 10%, > 10%)	0.273	1.479	0.734-2.981
Multivariate			
Sex (male, female)	0.017[a]	0.240	0.074-0.776
Age (< 53 yr, ≥ 53 yr)	0.075	0.538	0.272-1.065
Serum AFP level (< 30 μg/L, ≥ 30 μg/L)	0.343	1.476	0.661-3.296
HBsAg (positive, negative)	0.515	1.731	0.332-9.026
Tumor size (≤ 2 cm, > 2 cm)	0.711	1.535	0.159-14.827
Histological grade (well, moderately, poorly differentiated)	0.298	1.462	0.715-2.993
Liver cirrhosis (present, absent)	0.858	0.928	0.408-2.111
T classification (T1-T4)	0.001[a]	2.119	1.347-3.336
Wnt5a (low, high)	0.020[a]	0.288	0.101-0.824
Ror2 (low, high)	0.144	0.509	0.205-1.259
β-catenin (normal, abnormal)	0.013[a]	3.233	1.286-8.130
Ki-67 (mitosis ≤ 10%, > 10%)	0.494	0.839	0.507-1.387

[a]$P < 0.05$ *vs* over survival. AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; Wnt5a: Wnt member 5a; Ror2: Receptor tyrosine kinase-like orphan receptor 2

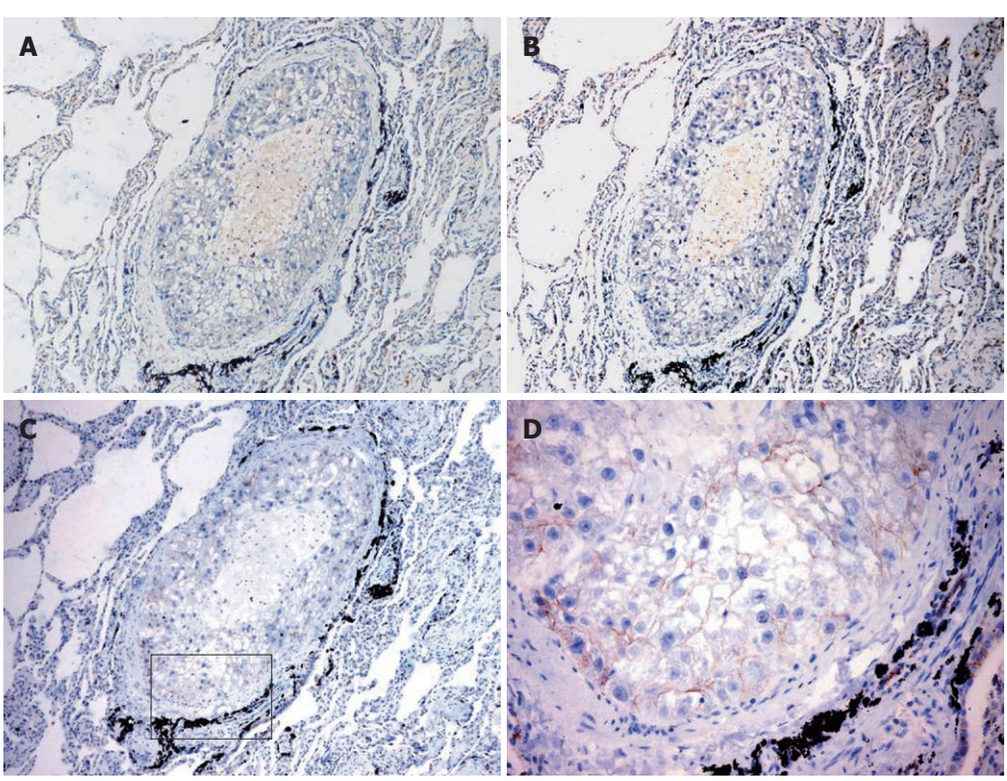

Figure 4 Immunohistochemical staining for Wnt member 5a (A), receptor 2 (B), β-catenin (C, D) in lung metastasis tissues. Original magnification: 400 × in D; 100 × in the others.

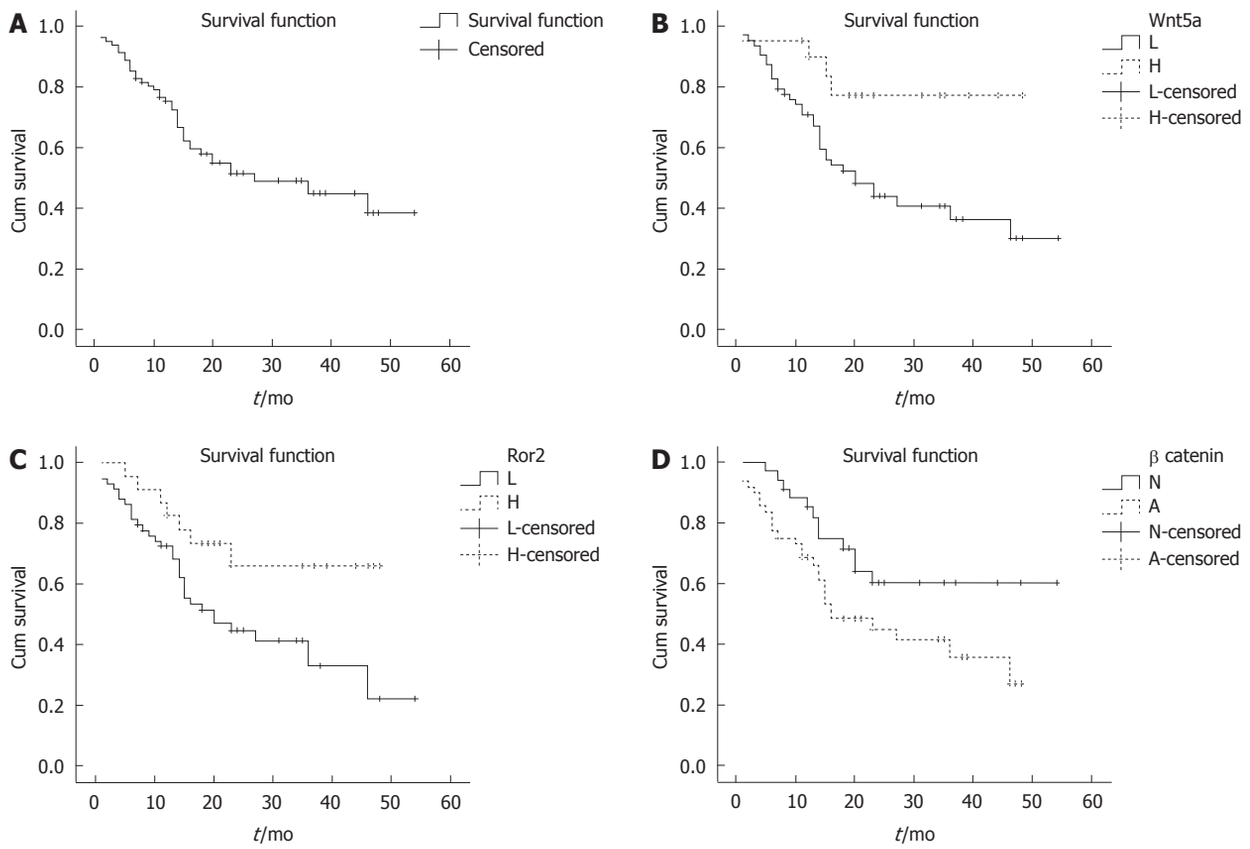

Figure 5 Survival curves of 82 hepatocellular carcinoma patients. A: Overall survival curves of 82 hepatocellular carcinoma (HCC) patients; B: Survival curves of 82 HCC patients with tumors expressing low or high levels of Wnt member 5a (Wnt5a) (log-rank test, $P = 0.016$); C: Survival curves of 82 HCC patients with tumors expressing low or high levels of receptor 2 (Ror2) (log-rank test, $P = 0.007$); D: Survival curves of 82 HCC patients with tumors expressing low or high levels of β-catenin (log-rank test, $P = 0.045$). L: Low expression; H: High expression; N: Normal expression; A: Abnormal expression.

survival. However, a significant correlation between the expression levels of Ror2 and overall survival ($P = 0.144$), serum AFP and overall survival ($P = 0.343$) were not demonstrated.

DISCUSSION

Consistent with previous reports[23,24], in this study immunohistochemical analysis showed that the loss of Wnt5a protein expression in HCC tumors frequently occurred in patients with HCC (71%-81%), and this also correlated with increased AFP and poor histologic grade. Wnt5a may act as a tumor suppressor gene in the development of HCC. Similar results were obtained in colon carcinoma, breast cancer and thyroid carcinoma[17,18,35,36]. We also performed a survival analysis for 82 patients with HCC. Our results demonstrated that HCC patients with low expression of Wnt5a had a poorer prognosis than those with high Wnt5a expression, and Wnt5a was an independent prognostic factor for HCC.

Recent studies have indicated that the upregulation of Wnt5a was associated with tumor invasiveness and metastasis in metastatic melanoma, gastric cancer, and non-small-cell lung carcinoma[17-19]. Wnt5a was expressed predominantly in the metastatic but not primary lesions of metastatic melanoma[34]. Therefore, three cases with lung metastasis of HCC were recruited in the present study. Immuno- histochemical analysis with anti-Wnt5a antibody showed that Wnt5a was not expressed in either primary or metastatic lesions, which confirmed our hypothesis that Wnt5a acts as a tumor suppressor gene in HCC. These observations suggested that the complex Wnt5a-regulated signal pathways and the functional role of Wnt5a depends on cell type as well as stimulus factors during the development of HCC tumor.

Previous reports showed that Ror2 shared a similar structure with the Wnt receptor[25]. Mikels *et al*[25,37] revealed that Wnt5a suppressed Wnt/β-catenin activity *via* the Ror2-mediated signal pathway, and confirmed that the Ror2 receptor required tyrosine kinase activity to mediate Wnt5A signaling. He *et al*[38] demon- strated that Wnt5a levels correlated with those of Ror2 during mammalian palate development. Similar to Wnt5a, Ror2 plays different roles in different human tumor tissues. There is evidence that the enhanced expression of Ror2 is associated with tumor invasiveness and metastasis in metastatic melanoma, renal cell carcinoma, and squamous cell carcinoma[29-31]. In contrast, the mRNA and protein expression of Ror2 was reduced in colon cancer tissues compared with adjacent nontumorous liver tissue, which might be due to the hypermethylated Ror2 promotor[32].

Our current study showed that *Ror2* gene transcrip-

tion and protein translation were both suppressed in tumor tissues of HCC as compared with tissue adjacent to the tumor. This reduced expression of Ror2 in tumor tissues was correlated with decreased Wnt5a expression ($P < 0.001$), a high Ki-67 LI, increased AFP, high differentiation, and poor prognosis. The consistency of Wnt5a and Ror2 expression in tumor tissues as well as in lung metastasis of HCC implies that Ror2 may be active downstream of Wnt5a and participate in the regulation of the noncanonical Wnt signal pathway. Moreover, the mRNA and protein expression of Ror2 is increased in chronic hepatitis livers and is greatly enhanced in cirrhotic livers as compared with normal liver tissues, suggesting Ror2 may play important roles in regulation of cell repair. The expression of Ror2 is reduced in tumor tissues and is associated with poor prognosis, indicating the impaired regulatory effect of Ror2 in cells, and Ror2 may also serve as an anti-tumor gene. In addition, in this present study, the mRNA expression of Ror2 was decreased in highly differentiated HCC as compared with moderately or poorly differentiated HCC ($P < 0.05$), whereas similar results were not obtained in the protein expression of Ror2. The underlying mechanism needs to be further elucidated. However, due to the limited sample size in highly differentiated HCC (3 cases), future study will be continued by enlarging the sample size.

β-catenin is recognized as the key mediator in the canonical Wnt signal pathway. Evidence indicates that Wnt5a inhibits the abnormal expression of β-catenin through the Ror2-mediated pathway[25,39,40]. The involvement of β-catenin in tumorigenesis has been intensively researched. In colon carcinoma, the nuclear localization of β-catenin induced by gene mutation contributes to tumorigenesis. However, in HCC associated with HBV infection, β-catenin mutations are rarely seen, and β-catenin mainly accumulates in the cytoplasm[41,42]. Consistent with these observations, statistically reduced membrane expression and elevated cytoplasmic expression of β-catenin were detected in HCC tumor tissue, compared with the β-catenin expression in cell membranes in the adjacent liver tissue. Among 85 HCC cases, 6 exhibited condensed nuclear staining of β-catenin, suggesting that this protein is involved in the development of HCC. Nevertheless, we could not rule out the possibility that the loss of Wnt5a and Ror2 protein expression may decrease β-catenin degradation, which contributes to disease progression. Additionally, the lining shape of β-catenin expression in cell membranes was observed in lung metastasis of HCC, which was different from their cytoplasmic expression in the primary lesion. Since β-catenin not only acts as the key mediator of the canonical Wnt signal pathway, but also binds to E-cadherin and together they contribute to the cell adhesion and migration process[43], we hypothesize that the lung metastasis expression of β-catenin benefits the accumulation and adhesion of tumor cells in metastatic lesions.

In summary, in patients with chronic hepatitis or cirrhosis, loss of Wnt5a and Ror2 protein expression in HCC tumor tissue frequently occurs during the progression of HCC and is associated with patient prognosis. We hypothesize that Wnt5a acts upstream of Ror2. Wnt5a and Ror2 synergistically execute an anti-tumor effect during the development of HCC. The decreased expression of Wnt5a and Ror2 in HCC tissues may be directly or indirectly correlated with the abnormal activity of β-catenin. It is possible that the Wnt5a-mediated noncanonical Wnt signal pathway and the β-catenin-mediated canonical signal pathway contribute to the pathogenesis and progression of HCC. These critical mediators may be novel promising targets for gene therapy. Our study showed that HCC patients with reduced Wnt5a and Ror2 expression had poorer prognosis, indicating that protein expression of Wnt5a and Ror2 might be used as clinicopathological biomarkers for prognosis of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Understanding the molecular biological features of HCC is necessary for early diagnosis and better prognosis. The potential role of Wnt member 5a (Wnt5a) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) in human HCC is receiving increasing attention.

Research frontiers

Recent work in a wide of human tumors has indicated that Wnt5a and Ror2 have a critical role in malignant progression. However, little is known about the association of Wnt5a expression with Ror2 and canonical Wnt in HCC. In this study, the authors demonstrate that Wnt5a, in conjunction with Ror2 and β-catenin, may take part in the progression of HCC.

Innovations and breakthroughs

The loss of Wnt5a and Ror2 protein expression in HCC tumor tissue frequently occurs during the progression of HCC and is associated with patient poor prognosis. Wnt5a and Ror2 synergistically execute an anti-tumor effect during the development of HCC. The loss of Wnt5a and Ror2 protein expression was shown to be associated with abnormal β-catenin expression. This is the first study to report an association of Wnt5a expression with Ror2 and β-catenin in HCC.

Applications

The study results suggest that protein expression of Wnt5a and Ror2 may be used as clinicopathological biomarkers for prognosis of HCC.

Terminology

Wnt5a is a non-canonical member of the Wnt family of secreted glycoproteins that acts through the family of frizzled G-protein-coupled receptor, Ror2, to mediate important events during development and cancer.

Peer review

This paper reported that the loss of Wnt5a and Ror2 protein expression in HCC was associated with poor patient prognosis. Based on reduction in tumors, the authors conclude these markers could be tumor suppressor genes and good prognostic markers for HCC patients. The work is purely descriptive and relevance to clinical practice is significant.

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BRIEF ARTICLE

Chronic hepatitis C: Treat or wait? Medical decision making in clinical practice

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Abstract

AIM: To analyzes the decision whether patients with chronic hepatitis C virus (HCV) infection are treated or not.

METHODS: This prospective cohort study included 7658 untreated patients and 6341 patients receiving pegylated interferon α2a/ribavirin, involving 434 physicians/institutions throughout Germany (377 in private practice and 57 in hospital settings). A structured questionnaire had to be answered prior to the treatment decision, which included demographic data, information about the personal life situation of the patients, anamnesis and symptomatology of hepatitis C, virological data, laboratory data and data on concomitant diseases. A second part of the study analyzes patients treated with pegylated interferon α2a. All questionnaires included reasons against treatment mentioned by the physician.

RESULTS: Overall treatment uptake was 45%. By multivariate analysis, genotype 1/4/5/6, HCV-RNA ≤ 520 000 IU/mL, normal alanine aminotransferase (ALT), platelets ≤ 142 500/μL, age > 56 years, female gender, infection length > 12.5 years, concomitant diseases, human immunodeficiency virus co-infection, liver biopsy not performed, care in private practice, asymptomatic disease, and unemployment were factors associated with reduced treatment rate. Treatment and sustained viral response rates in migrants (1/3 of cohort) were higher than in German natives although 1/3 of migrants had language problems. Treatment rate and liver biopsy were higher in clinical settings when compared to private practice and were low when ALT and HCV-RNA were low.

CONCLUSION: Some reasons against treatment were medically based whereas others were related to fears, socio-economical problems, and information deficits both on the side of physicians and patients.

Key words: Hepatitis C virus; Interferon, Ribavirin; Liver cirrhosis; Migrants; Treatment barrier

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INTRODUCTION

Approximately 170 million humans worldwide are estimated to have a chronic hepatitis C virus (HCV) infection including 400 000 in Germany[1,2]. More than 20 % of these patients will progress to cirrhosis, hepatocellular carcinoma, liver transplantation or death[3,4]. Therefore, all patients are candidates for antiviral therapy[5]. Its benefits need to be determined based on the individual's disease stage and on the likelihood of adherence and success[5,6]. Probably only 20 % of HCV-infected subjects know of their infection[3]. This diagnostic deficit is caused by various factors; e.g., physicians do not follow guidelines to screen for HCV infection when alanine aminotransferase (ALT) is elevated[7,8]. In addition only 11%-41% of known infections are treated[9-12]. Only some reasons for this therapeutic deficit have been identified including comorbidity, drug abuse and psychosocial factors[9,12-15]. Considering that therapy cures the disease in 50% of patients, treatment rate should be increased. The present study evaluates which factors influence the treatment decision in daily German practice.

MATERIALS AND METHODS

The study which is ongoing was started in March 2003; the present data analyzes the treatment decision in patients included between March 2003 and May 2008. Throughout Germany 434 physicians (377 in private practice and 57 in hospital settings) contributed a mean number of 35 patients with chronic hepatitis C. The study included only one academic center. Basic data of the cohort have been published[16] and are only briefly mentioned here. The study was approved by health authorities and ethical committees. Due to its observational character it did not affect individual medical decisions. A structured questionnaire had to be answered prior to the treatment decision; a second part of the study analyzes patients treated with pegylated interferon α2a (Pegasys®, Roche Pharma AG) and ribavirin. This part is not fully analyzed here; only those aspects are analyzed which are relevant to the treatment decision. All questionnaires included reasons against treatment mentioned by the physician. After July 2004 questionnaires also asked why patients denied therapy (n = 7658). Language skills were assessed after January 2006. Fibrosis was staged according to Desmet and Scheuer from F0 to F4[17]. Among the total 15 137 patients 7658 subjects did not receive any treatment ("untreated patients") while 6341 received pegylated interferon α2a and ribavirin ("treated patients") and 1138 alternative treatments. Details on alternative therapies (92.5% silymarin, 2.8% ursodesoxycholic acid, 4.9% other interferons) are not given because their characteristics were similar to the group receiving pegylated interferon α2a/ribavirin. Thus, in the following text the total cohort consists of 13 999 patients separated by the treatment decision into "treated patients" (n = 6341) and "untreated patients" (n = 7658). Specific procedures were not mandatory for inclusion except for documentation of chronic hepatitis C. There were no exclusion criteria except for patients below age 18 years and those with Child B/C cirrhosis. Thus, the study represents a real life scenario of a rather unselected cohort including a significant fraction of all patients diagnosed with hepatitis C in Germany.

Table 1 Demographic data and basic characteristics

Characteristics	Not treated (n = 7658)	Treated (n = 6341)
% of the 13 999 patients	55.7	45.3
Genotypes 1/4/5/6 (%)	69.8	59.4
Genotypes 2/3 (%)	30.2	40.6
Age (yr, median)	44.0	41.0
BMI (kg/m^2, median)	24.2	24.3
Gender (male %)	56.6	61.1
Regulär employment (%)	35.3	50.2
Infection length (yr, median)	11.0	10.0
Ultrasound performed (%)	76.8	87.6
Liver biopsy performed (%)	12.8	30.2
Fibrosis score F 0-1	72.8	58.6
Fibrosis score F 2-4	27.2	41.4
Active drug or alcohol abuse (%)	28.3	13.8
HIV co-infection (%)	6.7	3.7
Psychiatric disease (%)	14.8	9.2
Severe language problems (%)	9.6	10.0
Initial HCV-RNA (IU/mL, median)	482 500	500 000
ALT (U/L, median)	61.0	78.0
Thrombocytes (/μL, median)	217 000	218 000
At least on concomitant disease (%)	62.3	42.6

BMI: Body mass index; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; ALT: Alanine aminotransferase.

Statistical analysis

For continuous variables, receiver operating characteristic analyses estimated the best cut-off point for treatment decision; these cut-off points were 56 years for age, 520 000 IU/mL for basal HCV-RNA, ⩾ one concomitant disease, ⩾ 12.5 years for infection length, and 142 500/μL for platelets. Categorical variables were used for continuous variables using these cut-off points. Association of various factors with treatment decision and sustained virological response (SVR = negative HCV-RNA 24 wk after end of therapy) were analyzed in an

Table 2 Treatment and sustained virological response rates in various subgroups

				Fischer's exact test, two-sides *P* value	
	Treatment rate %	SVR %	Number	Treatment rate	SVR
Total	45.3	49.6	13 999		
Genotypes 1/4/5/6	41.4	42.7	9114	< 0.0001	< 0.0001
Genotypes 2/3	52.7	59.8	4885		
Clinical setting	63.9	49.8	1298	< 0.0001	NS
Private practice	43.4	49.6	12 701		
Male	47.2	47.9	8214	< 0.0001	< 0.01
Female	42.6	52.3	5785		
Age ⩽ 56 yr	49	51.3	11 497	< 0.0001	< 0.0001
Age > 56 yr	28.2	36.7	2502		
BMI ⩽ 23 (kg/m^2)	44.3	51.8	4762	< 0.01	< 0.05
BMI > 23 (kg/m^2)	46.9	48.6	8846		
No employment	38.9	47.3	8113	< 0.0001	< 0.001
Regular employment	54.1	52	5886		
Bad German language skills	47	52.5	824	NS	NS
Good German language skills	45.8	47.8	7565		
Migrants	53.3	52.6	2663	< 0.0001	< 0.0001
German natives	41.7	45.4	5465		
Infection length ⩽ 12.5 yr	62.8	51.6	3639	< 0.0001	< 0.01
Infection length > 12.5 yr	37.2	48	3165		
Ultrasound not performed	30.7	47.5	2568	< 0.0001	NS
Ultrasound performed	48.6	50	11 431		
Liver biopsy not performed	39.9	50.1	11 100	< 0.0001	NS
Liver biopsy performed	66.1	48.5	2899		
Fibrosis scores F0-1	60.9	52.4	1766	< 0.0001	< 0.01
Fibrosis scores F2-4	74.6	44.1	1017		
Clinical symptoms absent	42.2	47.8	4430	< 0.0001	NS
Clinical symptoms present	46.7	50.4	9569		
No concomitant disease	55.7	51.8	6527	< 0.0001	< 0.0001
At least one concomitant disease	36.2	46.8	7472		
Psychiatric disease absent	46.9	49.8	12 281	< 0.0001	NS
Psychiatric disease present	34.1	48.4	864		
Active drug or alcohol abuse absent	49.9	49.7	10 960	< 0.0001	NS
Active drug or alcohol abuse present	28.7	49.4	3039		
HIV co-infection absent	46.1	50	13 254	< 0.0001	< 0.01
HIV co-infection present	31.4	39.3	745		
Good quality-of-life	43.8	49.5	11 348	< 0.0001	NS
Reduced quality-of-life	51.8	50.1	2651		
ALT normal (< 50 U/L for men, < 30 U/L for women)	34.8	50.8	3297	< 0.0001	NS
ALT elevated (U/L)	49.6	49.7	10 105		
Thrombocytes ⩾ 142 500 /μL	48	51.6	11 284	< 0.0001	< 0.0001
Thrombocytes < 142 500 /μL	38.9	36.2	1816		
HCV-RNA ⩽ 520 000 IU/mL	45.4	54.8	6810	< 0.0001	< 0.0001
HCV-RNA > 520 000 IU/mL	49.7	43.3	5904		
No concomitant disease	55.7	51.8	6527	< 0.0001	< 0.0001
At least one concomitant disease	36.2	46.8	7472		
HIV co-infection absent	46.1	50	13 254	< 0.0001	< 0.01
HIV co-infection present	31.4	39.3	745		

SVR: Sustained virological response; BMI: Body mass index; HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; NS: Not significant.

univariate fashion using Fisher's exact test. Only those variables which were significant in the univariate analysis were included in the multivariate analysis.

RESULTS

Effects of various factors on treatment rate by univariate analysis

Basic characteristics of treated *vs* untreated patients are shown in Table 1. Many characteristics were similar for genotypes 1 (n = 8625), 4 (n = 440), 5 (n = 22) and 6 (n = 27) and for genotypes 2 (n = 1000) and 3 (n = 3885) (data not shown); thus, further analyses were done in two subgroups, i.e., genotypes 1/4/5/6 *vs* 2/3. Table 2 summarizes treatment and SVR rates in the total cohort (45.3% and 49.6%, respectively) and in treated *vs* untreated patients.

By univariate analysis reduced treatment uptake and reduced SVR were seen in these groups: (1) genotypes 1/4/5/6 *vs* 2/3; (2) age > 56 years *vs* ⩽ 56 years; (3) platelets ⩽ 142 500/μL *vs* > 142500/μL; (4) disease duration >12.5 years *vs* ⩽ 12.5 years; (5) human im-

Table 3 Treatment and sustained virological response rates *vs* socio-economic problems and concomitant diseases

Characteristics	Treatment rate %	SVR %	*n*
Drug abuse absent and employed without psychiatric disease or HIV co-infection	58.2	52.7	4382
Drug abuse absent and employed without psychiatric disease	58.2	52.4	4560
Drug abuse absent and employed	57.1	52.6	4929
Drug abuse absent	49.2	49.6	10 839
Drug abuse present	32.0	49.9	3160
Drug abuse present and unemployed	29.1	51.6	2203
Drug abuse present and unemployed with psychiatric disease	25.1	50.8	470
Drug abuse present and employed with psychiatric disease and HIV co-infection	7.1	0.0	56

HIV: Human immunodeficiency virus.

munodeficiency virus (HIV)/HCV co-infection *vs* HCV mono-infection; (6) presence *vs* absence of concomitant diseases; (7) German natives *vs* migrants; and (8) absence *vs* presence of regular employment.

Treatment uptake was reduced but SVR was higher in the following groups: (1) women *vs* men; (2) fibrosis F0-1 *vs* F2-4; and (3) basal HCV-RNA > 520000 IU/mL *vs* ≤ 520 000 IU/mL.

Treatment uptake was reduced while SVR was similar in the following groups: (1) normal *vs* elevated ALT; (2) good *vs* reduced quality of life; (3) treatment in private practice *vs* clinical setting; (4) presence *vs* absence of psychiatric disease; (5) presence *vs* absence of alcohol or drug abuse; and (6) liver biopsy (and ultrasound) not performed *vs* performed.

History of i.v. drug abuse was the most frequent mode of infection (44.6%) followed by history of blood transfusions (17.0%). By multivariate analysis infection mode did influence neither treatment uptake nor SVR (data not shown). In the total cohort only 20.7 % of patients had a liver biopsy. Biopsy was done more often in genotypes 1/4/5/6 when compared to genotypes 2/3 (23.6% *vs* 15.3%, $P < 0.001$) and in patients with elevated ALT (75.4% had elevated ALT) when compared to those with normal ALT (21.6% *vs* 18.4%, $P < 0.05$). Biopsy rate was three-times higher in hospital settings when compared to practioners (53.4% *vs* 17.4%, $P < 0.001$). Alcohol or drug abuse was a frequent treatment barrier in particular in patients with psychiatric diseases or HIV co-infection and in jobless people (Table 2). Treatment rates were similarly low in drug abusers with or without substitution (data not shown). Patients with alcohol or drug abuse refused therapy less often compared to patients without abuse (50.2% *vs* 67.9%, $P < 0.001$). Thus, the decision not to treat was made primarily by the physician. About 1/3 of all patients were migrants among whom 1/3 had severe language problems. Nevertheless, treatment and SVR rates were higher in migrants than in German natives while language problems did not affect treatment and SVR rates. Treatment uptake decreased with an increasing number of socio-economical and psychiatric problems; HIV infection on top of other problems reduced treatment uptake to 7 % (Table 3). SVR was unaffected even by presence of several socio-economical problems but was drastically reduced when there was a HIV co-infection on top of other problems.

Multivariate regression analysis

Gender, age, genotype, HCV-RNA, ALT, platelets, symptoms, infection length, occupational status, concomitant diseases, HIV co-infection, alcohol and drug abuse, performance of liver biopsy and ultrasound, and quality-of-life significantly affected the treatment decision in the multivariate analysis (Figure 1). In patients with genotypes 1/4/5/6 the same factors as for the total cohort affected the treatment decision except for presence of symptoms; in patients with genotypes 2/3 the same factors as for the total cohort affected the treatment decision except for symptoms, platelets, employment, and performance of liver biopsy (data not shown). SVR was associated with various factors in the univariate analysis (Table 2). By multivariate analysis SVR was associated only with gender, genotype, HCV-RNA, age, platelets, symptoms, employment and HIV co-infection (data not shown).

Analysis of specific reasons against treatment

The analysis looked at reasons mentioned by physicians and patients (Figure 2). The patients' wish was the most common reason against treatment (62.9 %). Among these patients lack of understanding the need of therapy, fear of side-effects, and problems with family and job were frequent reasons. Fear of side-effects was mentioned more often in women than in men (29.9% *vs* 18.8%, $P < 0.001$). Alcohol or drug abuse and concomitant diseases (most commonly depression) were also frequent treatment barriers. Among patients who did not see a need for therapy reasons included lack of liver disease, symptoms, fibrosis and bad prognosis as well as normal ALT. In patients with normal ALT minor disease activity was mentioned by the physician as a reason to wait in 24.1% whereas this reason was mentioned in only 6.6% when ALT was elevated ($P < 0.001$). In contrast, a similar percentage of patients mentioned the lack of disease activity as a treatment barrier irrespective of whether ALT was normal or elevated (27.1% *vs* 24.4%; NS). In patients with a HCV-RNA ≤ 520 000 IU/mL minor disease activity was mentioned by the physician as a reason to wait in 15.8% whereas this reason was mentioned in only 6.7% when HCV-RNA was > 520 000 IU/mL ($P < 0.01$). The percentage of patients mentioning lack of disease activity as a treatment barrier was similar when looking at high or low HCV-RNA (data not shown). In patients who had liver biopsy minor disease activity was mentioned by the

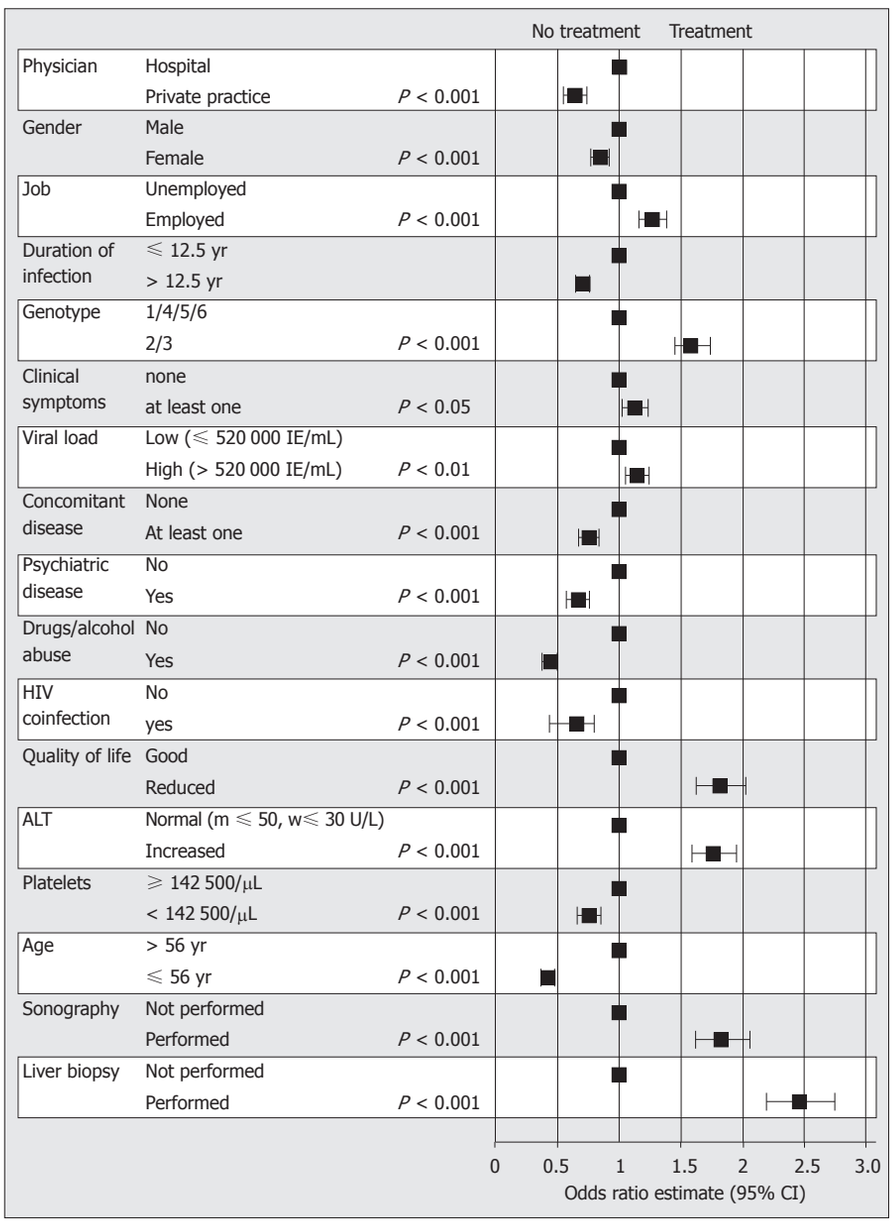

Figure 1 Multivariate regression analysis of treatment rates *vs* various factors. HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase.

physician as a treatment barrier in 21.4 % whereas this reason was mentioned in only 10.3 % of patients without a liver biopsy ($P < 0.01$). Patients mentioned fear of side effects and lack of understanding the need for therapy less often when treated in hospital settings as compared to private practice (18.5% *vs* 24.1% and 17.4% *vs* 25.9%, $P < 0.01$, respectively). In patients with drug/alcohol abuse, this abuse was the main treatment barrier mentioned by physicians (48.1 %). In contrast, patients with abuse refused therapy less often than those without (50.2% *vs* 67.9%, $P < 0.001$). In HIV co-infection concomitant diseases and drug/alcohol abuse were more frequent treatment barriers than in mono-infection (25.0% *vs* 16.6% and 25.2% *vs* 16.4%, $P < 0,01$). HIV co-infected patients refused therapy less often than mono-infected patients (59.1% *vs* 63.2%, $P < 0.05$). Similarly, in patients with psychiatric diseases, the psychiatric disease was the main treatment barrier (46.2%); among patients with psychiatric disease drug and alcohol abuse was another common barrier (24.5% *vs* 15.7% in patients without psychiatric disease; $P < 0.001$). Older age was associated with a reduced treatment rate (49.0% *vs* 28.2% in patients ⩽ 56 years *vs* patients > 56 years) (Table 2; Figure 1); in patients aged between 65 and 70 years treatment rate was 26.3% (158/600) and thus similar to the rate of 28.2% seen at ages > 56 years.

DISCUSSION

Treatment uptake in the present cohort (45%) is one of the highest reported in the literature. Since the cohort included a significant fraction of all HCV-infected patients in Germany, the high treatment rate is probably not due to selection bias. In the literature treatment uptake

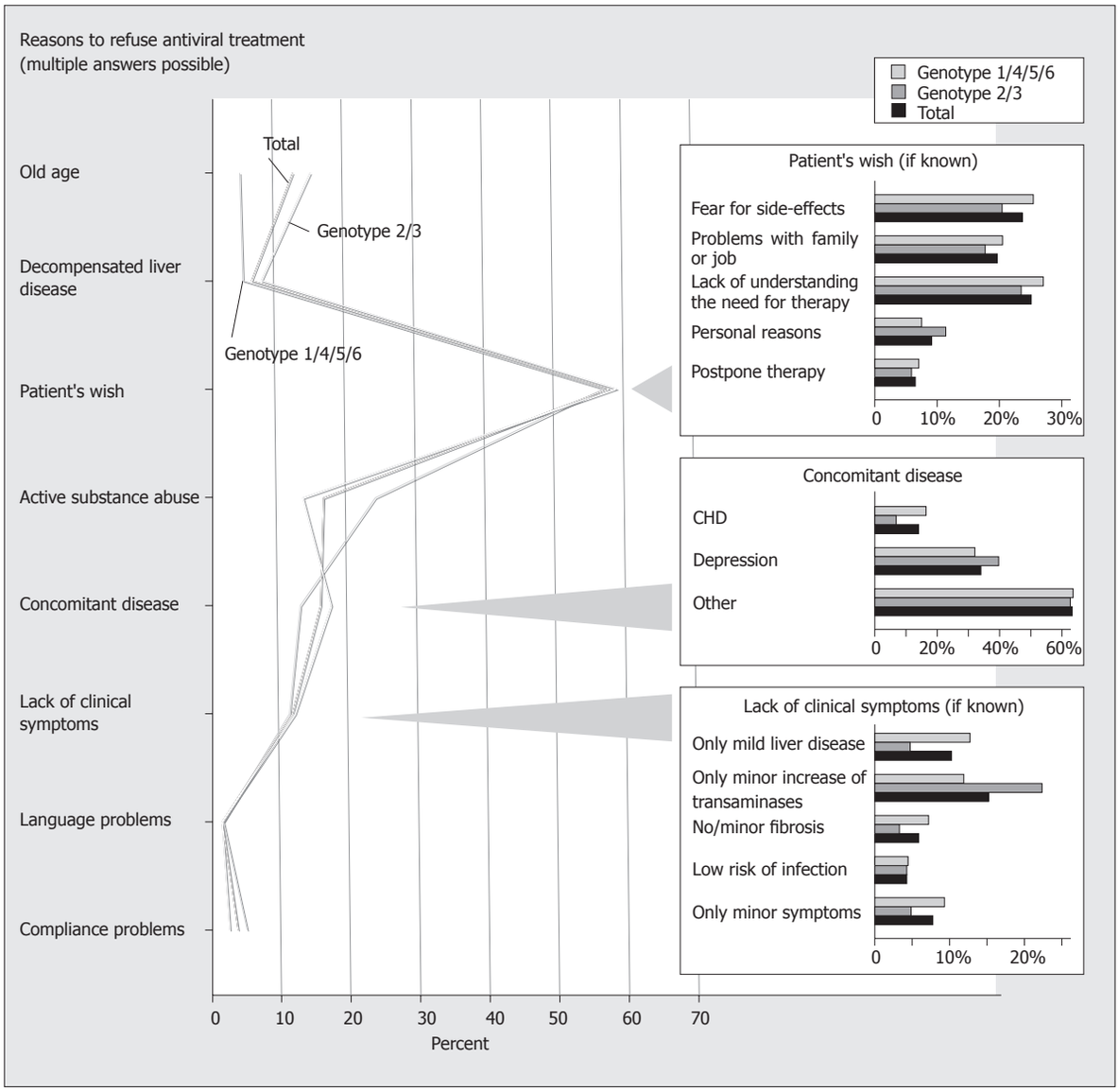

Figure 2 Reasons to refuse antiviral treatment.

tends to decrease with increasing number of subjects studied[9,12-15,18] with the lowest rate of 12% reported for the largest group of subjects studied[15]. There is little preselection in the present cohort; only patients with Child B/C cirrhosis were excluded as well as those under age 18 years. The present study did not include a relevant number of academic centers where most previous studies had been done. The community-based character of the present cohort incorporating 434 physicians and hospitals throughout the country reflects daily life in Germany probably better than looking at academic centres. However, one needs to keep in mind that most of the 434 physicians were not general practioners, but gastroenterologists or at least physicians who treat hepatitis C. In general practioners treatment rates may be lower than the 45% reported here. In the general United States community only 11% of all HCV-infected subjects had been treated[15]. This low treatment uptake suggests that therapeutic deficits are located on level of the general practioner or the health care system itself[7,8]. Recent studies show that knowledge deficits and misperceptions are main treatment barriers[19-21]. A high treatment rate might therefore reflect good knowledge among physicians and patients. In Germany most physicians who treat hepatitis C in private practice are organized in the Association of German Gastroenterologists ("bng"). Via their association gastroenterologists have been involved in the development of national HCV guidelines[6,22]. Many of them are members of the national "hepatitis competence network". Recent studies have also shown that German patients with hepatitis C are well informed and better than patients with hepatitis B[23-25]. However, some practice aspects did not meet standards in the present cohort including the use of liver biopsy and interpretation of HCV-RNA values. Also, there were misperceptions among patients. Patients' refusal was a common treatment barrier in the present cohort and in previous studies[9-11]. One of the highest treatment rates (41%) was published by Delwaide *et al*[9];

in that study only 17% of patients declined therapy. Thus, a high treatment uptake may be associated with low rate of refusal by patients[9]. This association may partly be explained by information deficits. In some subgroups, e.g., in patients with HIV co-infection and those with drug and alcohol abuse, the decision against treatment was often made by the physician whereas patients were rather willing to receive therapy.

Genotype and viral replication are major factors for estimating the chance for SVR and are therefore considered in the treatment decision. Correspondingly treatment rate and SVR were higher for genotypes 2/3 when compared to genotypes 1/4/5/6. In accordance with most previous studies[5,11,15,22] older age was associated with both reduced treatment uptake and reduced SVR in the present cohort. These results are in contrast to a recent study[18] in which being elderly was not associated with a low SVR. Surprisingly, treatment rate was low in patients with low HCV-RNA. This is a paradox because SVR is low at high replication in the present study and in the literature[26-28]. Thus, there may be misperceptions that high viral load indicates bad prognosis. All evidence shows this is not the case[22,29,30]. Further analyses suggested that physicians (and not patients) carry this misperception.

For many years normal serum aminotransferases were a common treatment barrier because they were thought to indicate good prognosis and reduced efficacy of therapy. In the meantime it has been shown that up to 30% of patients with normal ALT have major fibrosis and that SVR is not associated with ALT as also seen in the present study[22,29-31]. Despite this data, treatment rate was markedly lower in patients with normal ALT when compared to those with elevated ALT. We have reported a similar misperception of ALT for the decision to do HCV antibody tests[8]; many physicians just tested for HCV infection if ALT was markedly increased although most infections were associated with normal or slightly elevated ALT. Thus, ALT values are overestimated both in diagnostic[8] and treatment decisions[9,12].

In contrast to academic trials, only 20% of patients had a liver biopsy in daily German practice. According to guidelines liver biopsy should be considered when the results will influence the treatment decision and in particular when treatment is not initiated[5,22]. However, treatment rate in patients with a liver biopsy was twice that seen in patients without a biopsy; according to guidelines it should be the other way around. Only a single previous study has also shown a positive association between performance of liver biopsy and treatment uptake[32]. It may be speculated that patients who refused liver biopsy may have a general problem to accept medical means. However, further analyses support other explanations. Biopsy rate in hospital settings was more than three-times higher than that in private practice. Although non-invasive means of assessing fibrosis are entering clinical routine, only a minority of community-based physicians use serum markers or sonographic stiffness in daily clinical routine as yet. Thus, physicians in private practice underestimate the value of liver biopsy more often than physicians in hospital settings. The lack of immediate availability of biopsy may explain the low biopsy rate among practioners. Also, treatment uptake was markedly lower for patients treated in private practice when compared to hospital settings. The analysis of specific reasons against treatment may partly explain this difference: patients mentioned fear of side effects and lack of understanding the need for therapy less often when treated in clinical settings when compared to private practice.

The treatment rate of HCV infection was considerably lower in HIV co-infected patients when compared to HCV mono-infection. Although SVR rates were also somewhat lower in co-infected patients, they were still in an acceptable range considering that end-stage liver disease is a common cause of death in HIV/HCV co-infection[33-35]. When compared with the literature the present rates of treatment and SVR (31% and 39%) look favorable. In other studies SVR ranged from 8% to 40% in co-infected patients[36-38]. Nevertheless HIV co-infection was a main treatment barrier also in the present cohort. Among co-infected patients drug and alcohol abuse as well as fear of side-effects were frequent treatment barriers. The present analysis also shows that HIV/HCV co-infected patients refused therapy less often than mono-infected patients; thus the low treatment rate is probably mainly caused by physicians and not by patients. In previous studies only 12%-33% of HIV co-infected patients initiated HCV therapy[36,39-40]; main barriers were non-adherence, patients' refusal, drug abuse and psychiatric problems. The present results demonstrate that the HIV infection on top of psychiatric and socio-economical problems may not only reduce treatment uptake but almost eliminates chances for SVR.

Recently it has been shown that HCV infection can successfully be treated in patients with drug and alcohol abuse and in those with HIV co-infection provided that there is a good management[35-38,41-43]. This is of great importance because alcohol abuse and co-infections accelerate fibrosis[34,35,44,45]. Although a history of drug abuse did not reduce treatment rate in the present cohort, active alcohol and drug abuse were associated with a markedly reduced treatment uptake as reported previously[10,11,14,15]; SVR was not affected by abuse. In 50% of abusers, physicians specified the abuse as the main treatment barrier. In contrast, patients with alcohol or drug abuse refused therapy less often than did patients without abuse. Thus, the decision not treat was made primarily by the physician. A survey of 320 American Society of Addiction Medicine physicians showed that even among these specialists only a minority were providing HCV treatment or willing to provide treatment[46]. Treatment rates are even lower in the general community and may approach values of less than 1 % in unselected drug addicts[47].

Treatment rate was lower in unemployed patients when compared to those with a job while SRV was similar between these groups. Since jobless people tend to have a low educational state, these results fit to recent United States data showing that psychosocial factors and low education were associated with reduced treatment up-

take[12,14,48]. In the present cohort 1/3 of HCV infected patients were migrants among whom 1/3 had severe language problems. Unexpectedly, treatment uptake was not lower but higher in migrants when compared to German natives. These results can not be explained easily. Along this line women had a lower treatment rate when compared to men in this cohort as well as in another previous study[10]. This is also unexpected because men have a lower use of medical services than women both in the United States[49] and in Germany[50]. Thus, good knowledge and care about health issues *per se* do not necessarily increase treatment uptake for hepatitis C.

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COMMENTS

Background

In recent surveys only 20% of hepatitis C virus (HCV)-infected subjects know of their infection and only 20% of the latter are treated. Considering that therapy cures the disease in 50% of patients, treatment rate should be increased.

Research frontiers

Bio-epidemiological research focuses to identify treatment barriers in patients with chronic hepatitis C. As yet only some reasons for the current large therapeutic deficit have been identified including co-morbidity, drug abuse and psychosocial factors. The present study evaluates which factors influence the treatment decision in daily German practice.

Innovations and breakthroughs

Treatment uptake in the present cohort (45%) is one of the highest reported in the literature. A high treatment rate usually reflects good knowledge among physicians and patients. In Germany many physicians who treat hepatitis C are members of the national "hepatitis competence network" which is aimed to implement practice guidelines in the broad medical community. Despite the obvious success of the German hepatitis competence network some practice aspects did not meet standards in the present cohort including the use of liver biopsy and interpretation of HCV-RNA and alanine aminotransferase (ALT) values. Liver biopsy and thus knowledge about fibrosis stage were too low in particular in patients treated in private practice and in those with normal ALT. Also, there were misperceptions among patients as their refusal was a common treatment barrier. Unexpectedly, therapy uptake was higher in migrants despite language problems. Some further reasons against treatment appeared medically based whereas others seemed to be based on fears, socioeconomical problems and information deficits both on the side of physicians and patients.

Applications

The present cohort study includes a significant fraction of all HCV-infected patients in Germany. The community-based character of the present cohort incorporating 434 physicians and hospitals throughout the country reflects daily life in Germany probably better than looking at specialized academic centres.

Terminology

Treatment barrier: Reasons why patients with chronic hepatitis C are not treated with antiviral drugs.

Peer review

This is an important paper with a large HCV patient cohort from Germany including both academic and non-academic centres detailing reasons for treating and not treating HCV.

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BRIEF ARTICLE

Celiac disease: Management of persistent symptoms in patients on a gluten-free diet

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Abstract

AIM: To investigate all patients referred to our center with non-responsive celiac disease (NRCD), to establish a cause for their continued symptoms.

METHODS: We assessed all patients referred to our center with non-responsive celiac disease over an 18-mo period. These individuals were investigated to establish the eitiology of their continued symptoms. The patients were first seen in clinic where a thorough history and examination were performed with routine blood work including tissue transglutaminase antibody measurement. They were also referred to a specialist gastroenterology dietician to try to identift any lapses in the diet and sources of hidden gluten ingestion. A repeat small intestinal biopsy was also performed and compared to biopsies from the referring hospital where possible. Colonoscopy, lactulose hydrogen breath testing, pancreolauryl testing and computed tomography scan of the abdomen were undertaken if the symptoms persisted. Their clinical progress was followed over a minimum of 2 years.

RESULTS: One hundred and twelve consecutive patients were referred with NRCD. Twelve were found not to have celiac disease (CD). Of the remaining 100 patients, 45% were not adequately adhering to a strict gluten-free diet, with 24 (53%) found to be inadvertently ingesting gluten, and 21 (47%) admitting non-compliance. Microscopic colitis was diagnosed in 12% and small bowel bacterial overgrowth in 9%. Refractory CD was diagnosed in 9%. Three of these were diagnosed with intestinal lymphoma. After 2 years, 78 patients remained well, eight had continuing symptoms, and four had died.

CONCLUSION: In individuals with NRCD, a remediable cause can be found in 90%: with continued gluten ingestion as the leading cause. We propose an algorithm for investigation.

Key words: Celiac disease; Non-responsive celiac disease; Refractory celiac disease; Gluten; Gluten-free diet

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INTRODUCTION

Celiac disease (CD) is induced by ingestion of gluten and related proteins with consequent intestinal injury and varied clinical manifestations. The defining feature is the expectation that the intestinal lesion improves with strict

exclusion of gluten from the diet. However, a proportion of individuals do not respond to a gluten-free diet (GFD), in terms of clinical or histological recovery. Early analysis has indicated that as many as 30% of individuals prescribed a GFD do not experience symptomatic improvement[1]. Non-responsive CD (NRCD) is defined as continued symptoms (including lethargy, abdominal pain and diarrhea) in patients on a GFD. There have been no recent studies to provide robust epidemiological data to assess the incidence of NRCD, although in clinical practice it is a common occurrence, based on the authors' experience and several publications[2-5]. The investigation of NRCD has been reported[6], however, there are no data on the management and longer term follow-up of these subjects. Most patients with CD experience a rapid symptomatic recovery with a strict GFD. In 30% of cases there may be a protracted (≥ 12 mo) or incomplete phase of mucosal recovery[7]. An arbitrary period of 6-12 mo on a GFD before reassessment has been suggested but the urgency of further investigation is often dictated by the severity of continued symptoms or clinical manifestations. In this context, we define NRCD as failure of expected symptomatic response to a GFD. Accordingly, NRCD is not intended to be a diagnostic term but rather a clinical description to allow a pragmatic and systematic approach to be followed to evaluate and investigate these patients. The practical management of NRCD depends on establishing a cause for continued symptoms. The commonest reason for persistent symptoms in a previous study of 55 patients was failure to comply with a GFD[6]. Imposition of a strict gluten-free dietary regimen appears to abolish symptoms in the majority of CD patients with continued symptoms[8].

Refractory CD (RCD) describes a distinct clinical entity and represents a subset of non-responsive patients. RCD is defined by symptomatic and persistent villous atrophy in patients despite a strict GFD[8]. RCD can be diagnosed after primary failure of GFD or occur as a secondary phenomenon in previously treated CD. It can be subdivided into types Ⅰ and Ⅱ. This clinical definition has been refined by the discovery that 80% of individuals with true RCD possess an abnormal population of intraepithelial lymphocytes (IELs) detectable in their small intestinal mucosa ($CD103^+$, intracellular $CD3^+$, $CD4^-$, $CD8^-$, surface $CD3^-$)[8]. These IELs may demonstrate a monoclonal T cell receptor (*TCR*)-γ gene rearrangement, detectable by polymerase chain reaction (PCR) analysis of biopsy specimens. The presence of this aberrant T cell phenotype has been termed type Ⅱ RCD (as opposed to type Ⅰ RCD in which this anomaly is not present). Studies have shown that type Ⅱ RCD is associated with a significantly greater mortality than type Ⅰ RCD; 41% *vs* 14% at 2 years[9]; 42% *vs* 4%[10] and 56% *vs* 7% 5-year mortality[11], with the major cause of death attributed to the development of enteropathy-associated T cell lymphoma (EATL). This is characterized by malignant lymphoid tissue with the same immunophenotype as described in type Ⅱ RCD. It has been postulated that the presence of this type Ⅱ RCD T cell phenotype may represent a cryptic T cell lymphoma. In 41 patients with RCD, over 50% developed EATL during a mean of 2 years follow-up[9]. Survival from EATL remains abysmal. Thus, there are compelling clinical reasons to investigate CD patients with continued symptoms despite a GFD, in order to establish a treatable cause or identify cases of RCD or intestinal lymphoma. NRCD and RCD may both be present with weight loss, diarrhea, or malabsorption; all of which warrant expeditious investigation.

MATERIALS AND METHODS

We maintain a prospective database of patients diagnosed with CD. We selected patients who were referred to our institution with a diagnosis of NRCD (defined as failure of expected symptomatic response to a GFD) between April 2002 and October 2003.

Initial evaluation included an appraisal of the original diagnosis of CD, history of symptoms (including lethargy, increased bowel frequency and weight loss), clinical examination, routine blood tests and assessment of dietary intake and GFD compliance. Patients were then investigated according to our usual clinical practice and subsequent findings; thus, some patients were investigated differently to others, however, all patients were followed for a minimum of 2 years; those who developed further symptoms were reinvestigated. Unless an obvious cause was immediately apparent, we undertook a further small bowel biopsy, which was performed by the authors to ensure a standard quality of biopsy specimen. Jumbo endoscopy forceps were used to obtain four samples that were carefully placed, mucosal surface upwards, onto paper to ensure optimal orientation.

Following standard preparation, histological examination was performed by our histopathology department, although in borderline or ambiguous cases, we often elected additionally to examine the slides within our department. An excess above 20 IELs per 100 enterocytes defined a pathological increase and villous atrophy was defined as being unequivocally present if the villous height to crypt depth ratio was below 2[12]. Direct visual comparison was made with any previous small intestinal specimens for the same patient. If there were any concerns regarding the validity of the diagnosis of CD, a gluten challenge was carried out. This involved ingestion of 10 g gluten (equivalent to four slices of white bread daily) for a minimum of 2 wk before repeat duodenal biopsy[13]. If colonoscopy was performed, random colonic biopsies were taken. In the diagnosis of microscopic colitis, we defined this condition as > 20 lymphocytes per 100 epithelial cells in the superficial colonic mucosa in patients with diarrhea[14].

Tests for small bowel bacterial overgrowth (SBBO) involved a lactulose hydrogen breath test. A positive test was indicated by an early rise in breath hydrogen >

20 ppm from baseline after ingestion of 10 g lactulose. We note the low sensitivity and specificity of breath tests for bacterial overgrowth, including hydrogen and labeled carbon tests[15]. In order to validate a diagnosis of SBBO, we additionally required that symptoms resolved following rotating antibiotic treatment (ciprofloxacin 250 mg bd for 2 wk followed by metronidazole 200 mg tds fortnightly for 4 mo).

Lactose intolerance was diagnosed on dietary exclusion alone as tests are also unreliable. Exclusion of dairy products carries no risk and, if symptoms resolve, is reliable in establishing a confident diagnosis of lactose intolerance. Non-invasive testing of pancreatic function was performed in a number of patients (pancreolauryl test). False positives may occur in CD[16], so the diagnosis could only be confirmed with symptomatic improvement with oral pancreatic supplements.

RCD was suspected in those with severe, symptomatic NRCD with demonstrated villous atrophy, particularly those with pronounced weight loss. Urgent and extensive investigation was arranged in these individuals. This included computed tomography scanning of the abdomen and pelvis, colonoscopy and small bowel imaging. Video capsule endoscopy was not routinely performed at the outset of this study, although this now forms part of our assessment of suspected RCD. If appropriate, serological testing for anti-enterocyte antibody was performed to exclude autoimmune enteropathy.

Additionally, tissue analysis for IEL immunophenotyping and PCR reaction amplification for TCR clonality were undertaken; DNA was analyzed by a series of multiplex PCR assays, which amplified *TCR* β and γ gene rearrangements. PCR primer sequences were those used by the Biomed-2 consortium and have been shown to detect clonal signals in approximately 95% of all T cell clonal cases[17,18].

The presenting symptoms, investigation process, results and outcome of subsequent management were recorded. We followed up patients for a minimum of 2 years and observed if patients remained symptom-free or suffered further relapses or related adverse events such as death or identification of malignancy. Any other tests deemed necessary, based on clinical history and examination, were performed, which resulted in a number of other diagnoses.

RESULTS

One hundred and twelve patients were referred to our center and underwent assessment for NRCD. The mean age of this group was 48.5 years (range 19-72 years) and 69% were female; CD had been diagnosed at a mean age of 31 years. The commonest presenting symptoms were diarrhea (65%), lethargy (43%), abdominal pain (27%) and weight loss (23%). The demographic details and clinical symptoms are shown in Table 1. The results are summarised in Figure 1

Twelve out of the 112 patients had been wrongly diagnosed with CD. Due to the doubt over the diagnosis, these 12 patients underwent gluten challenge and repeat biopsy which was normal in all cases. Additionally, anti-endomysial antibody (EMA) tests were all negative, although four patients had anti-gliadin antibodies detected. In four cases, initial duodenal biopsy had not been performed previously and diagnosis had been made based on dramatic reduction in symptoms with initial wheat exclusion. In the remaining eight, we were able to examine the original histology in five patients. Four of these were sufficiently normal to exclude CD in tandem with the subsequent negative gluten challenge. One patient did have villous atrophy on their original biopsy, which was felt to have been due to bacterial overgrowth, which had subsequently improved with antibiotic treatment. We were not able to examine previous specimens from three patients but the negative gluten challenge was deemed sufficient to exclude a diagnosis of CD. In total, six out of 12 patients had been previously shown to have supportive positive serology for CD in other institutions (mainly anti-gliadin antibody). Seven patients were diagnosed with irritable bowel syndrome; three with primary SBBO; and one each with anorexia nervosa and IgE-mediated wheat allergy. These individuals were subsequently removed from the analysis.

Table 1 Demographics and distribution of symptoms in 112 patients referred to our institution with continued symptoms on a gluten-free diet (%)

Male	31
Female	69
Mean age (yr)	48.5
Primary non-responsive	72
Secondary non-responsive	28
Mean years since diagnosis of CD (yr)	3 (range 1-12)
Diarrhea	65
Lethargy/fatigue	43
Abdominal pain	37
Weight loss	23
Nausea and vomiting	10
Symptoms of anemia	10
Two symptoms	49
Three symptoms	20

CD: Celiac disease.

Forty-five of the remaining 100 patients were found to be ingesting sufficient gluten to cause their symptoms. Of these, 24 were discovered to be consuming gluten accidentally, and 21 admitted poor compliance with aspects of their prescribed diet. In total, 37 (23/24 accidental group and 14/21 poor compliance group) patients underwent repeat duodenal biopsy in order to establish this information. Of these specimens, 33/37 were abnormal (Marsh Ⅲa-c) which assisted in correlating the continued ingestion with the persisting histological abnormalities.

The majority (28/37) proceeded to have a further duodenal sample taken that showed comparative improvement in all but one case. In this case, further gluten ingestion was admitted on further questioning. In summary, all 45 patients in this group reported symptomatic improve-

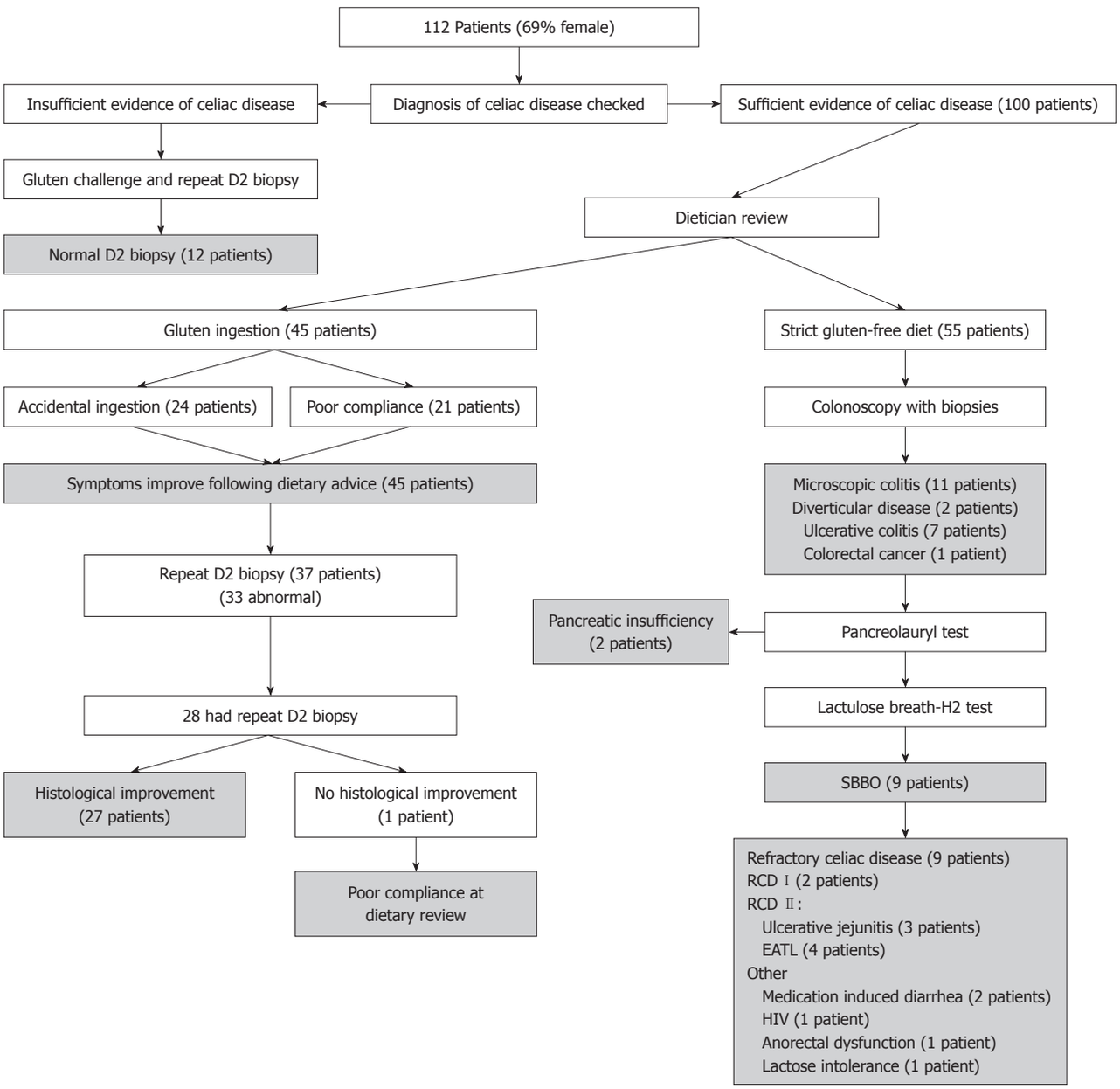

Figure 1 Flow chart showing the investigation and diagnoses of the patient cohort. RCD: Responsive celiac disease; **SBBO: Small bowel bacterial overgrowth;** EATL: Enteropathy-associated T cell lymphoma.

ment on a strict GFD, with 27/45 having demonstrable histological improvement.

Eleven patients were treated successfully for microscopic colitis. Diagnosis was made based on the presence of diarrhea and typical colonic histological features. All of these patients underwent simultaneous small bowel biopsy which was abnormal in 7/11 (64%) cases, mainly with an isolated intra-epithelial lymphocytosis. No alternative cause was established on enquiry or testing. These individuals were treated with a combination of mesalazine, loperamide, prednisolone and azathioprine (1-2.5 mg/kg). Five out of 11 required azathioprine for resolution of symptoms. Three patients suffered a relapse of diarrhea within 2 years; again treated successfully with oral steroids. When abnormal, patients had comparative improvement in their duodenal histology following resolution of symptoms. We performed a total of 75 colonoscopies in NRCD patients with diarrhea and found significant lymphocytic infiltration in 15. This included four patients defined as having RCD who did not show histological or clinical improvement with immunosuppressive treatment.

Nine patients were successfully diagnosed and treated for bacterial overgrowth with sustained resolution of symptoms. There have been two relapses both in the same patient within 2 years; responding on each occasion to further courses of antibiotic treatment (metronidazole and ciprofloxacin). Interestingly, one patient was found to have combined variable immunodeficiency as an underlying cause for bacterial overgrowth and was referred for immunoglobulin infusions as part of further management.

Ten patients had normal investigations (all had duodenal biopsy and colonoscopy). This group was reassured and treated symptomatically for irritable bowel syndrome. At review after 2 years, 5/10 had continued functional symptoms with no new positive investigations. One patient had been diagnosed empirically with lactose intolerance. The remaining four patients were symptom free.

Lactose intolerance was diagnosed in six individuals; all of whom had dramatic symptomatic resolution when a lactose-free diet was commenced. All of these patients had primary NRCD.

We identified seven patients with coexisting inflammatory bowel disease (IBD); all of whom were suffering from ulcerative colitis. The predominant pattern was proctitis in five patients, and two had sigmoid colitis. Six responded to 5-ASA therapies, and one required azathioprine to control their IBD. All remained well and no surgical intervention has been required at 2 years follow-up.

After initial assessment and duodenal biopsy, 20 patients were considered to have a high suspicion of RCD. All of these patients had weight loss and diarrhea and a history of positive correlative celiac serology. After exhaustive investigation and assessment according to the United European Gastroenterology Week guidelines[11] (median duration 5 mo), a firm diagnosis of RCD was made in 9/20 patients; all of whom had a raised IEL count (> 20 per 100 enterocytes). Furthermore, all had marked villous atrophy (Marsh Ⅲa-c). None of this group was found to have a positive anti-enterocyte antibody. An alternative and remediable explanation for symptoms was identified in 11 patients (seven continued gluten ingestion; three with bacterial overgrowth; and one with microscopic colitis). RCD may be divided into those without aberrant T cells (type Ⅰ) and those with aberrant T cells or ulcerative jejunitis (type Ⅱ)[11]. Of the nine refractory patients, seven had type Ⅱ RCD with positive clonality by γ TCR PCR. Three had ulcerative jejunitis; four were found to have or developed an enteropathy-associated intestinal lymphoma, two of whom have subsequently died, one from proven EATL and the other from suspected EATL (a post-mortem was refused by the relatives); both survived less than 1 year from diagnosis. The other two patients remain alive; one is on immunosuppressive medication and the other has been successfully treated with surgery. The remaining patients have continued to have symptoms over the follow-up period of 2 years (median 33 mo).

Of the two patients with type I RCD, one has died but we have no information available as to the precise cause of death, and the other patient has continued to have symptoms over the follow-up period of 2 years. In summary 3/9 (33%) patients diagnosed with RCD in our study have died.

Other diagnoses that were established are listed in Table 2. A diagnosis was only included if the symptoms were clearly attributable and symptomatic improvement occurred with appropriate treatment. Ten patients had more than one diagnosis established during the study period (median 33 mo). This was largely as a result of ongoing investigation for additional symptoms during the study period.

Table 2 Summary of established diagnosis in 100 patients referred to our center with non-responsive celiac disease

Diagnosis	*n*
Continued dietary gluten	45
Microscopic colitis	11
Bacterial overgrowth	9
Lactose intolerance	7
Inflammatory colitis	7
Irritable bowel syndrome	10
Refractory celiac disease	9
Type Ⅰ RCD	2
Type Ⅱ RCD	7
Anorexia nervosa	2
Pancreatic insufficiency	2
Diverticular disease	2
Medication-induced diarrhea	2
Combined variable immunodeficiency	1
Human immunodeficiency virus	1
Colorectal cancer	1
Anorectal dysfunction	1
Incorrect diagnosis of celiac disease	12

RCD: Responsive celiac disease.

Further assessment of patients' symptoms was conducted 2 years after their initial evaluation. Overall, four patients had died, with one from an unrelated cause. The vast majority (78%) reported being symptom-free at 2 years. A total of eight patients reported continued symptoms, with four describing them as moderate or severe. Those with continued symptoms included four diagnosed with RCD, two with irritable bowel syndrome and two with microscopic colitis. Ten patients could not be contacted.

In the 100 patients with NRCD, 73% had detectable anti-tissue transglutaminase (tTG) antibodies at varying titers. There was no statistical correlation between presence of antibodies, antibody titer and the established cause of NRCD. However, it was noted that 9/20 patients with RCD had positive celiac serological tests.

DISCUSSION

Evaluation of patients referred to us with continued symptoms on a GFD concluded that 12 out of 112 patients did not actually have CD. The diagnosis of CD might appear straightforward but this indicates that errors are still made in clinical practice. The main difficulties appear to be basing the diagnosis on serology alone; where available tTG and EMA should be tested because these are most sensitive and specific[19,20]. DQ2/8 HLA typing may be useful to exclude CD in patients when tTG is negative but villous atrophy is present, and there is doubt over the diagnosis. In this study, DQ2/8 was not performed given difficulty in availability; furthermore, it adds little in patients who have a positive tTG and villous atrophy. In experienced hands, serology testing is highly specific but,

there can be discordant results between different laboratories. The limitations of celiac serology have previously been reported[21]. Accordingly, duodenal biopsy remains mandatory for a clear diagnosis of CD to be made and this is supported by current recommendations. We feel it is important to reassess the initial biopsy, as failure to orientate the small intestinal mucosal biopsy can result in a false interpretation of villous atrophy.

When the diagnosis of CD is secure, investigation of continued symptoms yields a remediable cause in 90% of cases, with continued gluten ingestion as the leading diagnosis. This parallels the findings of a previous study in an NRCD group[6]. In our study, the commonest culprit for inadvertent intake was malted breakfast cereals, although beer, cooking sauces, pizza, and biscuits - the latter two of which were clearly labeled as containing gluten - were also identified as sources of continued gluten ingestion. The diagnosis of continued gluten ingestion was only accepted, if after dietary modification, the patients' symptoms were reported to have resolved at a later follow-up appointment. It is of interest that nearly half of those failing to adhere to a GFD were aware that their compliance was suboptimal but withheld this information at initial assessment. It appears that some CD patients are reluctant to acknowledge that a minor intake of gluten could account for their continued symptoms. It is therefore important that appropriate dietary advice is provided at the outset to avoid unnecessary investigation at a later date. Celiac societies have a useful role in advising on GFD. However, some patients in our study were following a recommended GFD but improved when certain "safe" foods were removed from their diet. There has been considerable debate as to the acceptable safe threshold for gluten in foods, with 200 ppm being initially recommended. Some individuals do appear to suffer ongoing symptoms with persistent duodenal injury, even with trace quantities of gluten ingested in certain foods. Therefore, a lower limit of 20 mg/kg (20 ppm) has been accepted for labeling of gluten-free foods with 100 mg/kg labeled as gluten-reduced. These regulations will be introduced in 2012[22]. We advise patients that appear to be exquisitely sensitive to traces of gluten to adhere to a wheat-free GFD. This involves avoidance of products that are made by extraction of wheat proteins from flour because this process is usually incomplete to some degree, with traces of residual gluten remaining.

The association of microscopic colitis has been reported in CD patients[6,13]. It has been postulated that the lymphocytic infiltrate is part of the same autoimmune pathogenesis that is seen in the small bowel and that this infiltrate improves with a GFD. Similarly, microscopic colitis appears to be linked with RCD, which again suggests an aberrant immunological process. In our study, we suggest that there may have been an overlap between the groups diagnosed with microscopic colitis and RCD. It may be difficult to differentiate the two conditions, especially when duodenal abnormalities are marked. We based our diagnosis on the predominant abnormality between colonic and duodenal histology, severity of clinical manifestations, and the response to treatment. In practice, once lymphoma has been exhaustively excluded, the management of resistant symptoms may be largely similar with recourse to immunosuppressive therapy. Treatment of microscopic colitis is currently suboptimal but overall, the natural history is benign. We do not attempt here to discuss the validity of treatments for microscopic colitis in CD; only that sustained symptomatic improvement was achieved in these cases. In our experience, a trial of oral mesalazine may prove sufficient, although this is frequently ineffective. Following this, moderate-dose oral systemic steroids (20 mg/d prednisolone) usually provides rapid complete symptomatic response. The dose should be tapered gradually, although in a few cases it may be necessary to maintain 5-7.5 mg/d; in such instances, azathioprine as a steroid-sparing agent should be considered.

SBBO is associated with CD and is probably underdiagnosed[23]. The mucosal abnormalities may theoretically disrupt the innate defenses of the small intestine and predispose to this condition. In our study, patients all responded to antibiotic therapy but relapse was common. A second longer course of rotating antibiotic therapy was prescribed, which appeared to eradicate symptoms in the long term. If suspected, the diagnosis can be difficult to confirm, either by duodenal aspiration or breath test because these tests have a low sensitivity and specificity[15]. The duodenal histology may be normal, abnormal or exhibit patchy changes that are difficult to detect[24]. Treatment may be reasonably advised empirically if this diagnosis is suspected[23]. Although there is minimal data from clinical trials, it is our practice to treat patients with ciprofloxacin 250 mg twice daily, rotating fortnightly with metronidazole 200 mg three times daily for 3 mo. Symptomatic response is assessed to determine success of treatment. Duration of treatment depends on the conviction that SBBO is the underlying course. In our experience, patients may require treatment with alternating antibiotics for up to 4 mo if symptoms are resistant or recur after discontinuation of a short course of empirical antibiotics.

Acquired lactose intolerance is widely recognized to be a potential problem in CD. Exclusion of dairy produce is often recommended in the first 3-6 mo of GFD to allow disruption of the brush-border disaccharidase activity to recover. IBD can coexist with CD, because both are common and not mutually exclusive. One study has previously reported an increased incidence of IBD in patients with CD compared with the general population[25]. Two patients in our cohort had evidence of concomitant pancreatic insufficiency. Abnormal exocrine function, as tested with fecal elastase, was demonstrated in 13 (42%) subjects in one series of 31 CD patients, although only in three was this clinically significant[26]. A trial of treatment with pancreatic supplements may be advisable in those in whom pancreatic insufficiency is suspected.

Continued symptoms in CD patients may be functional because the symptoms are often indistinguish-

able[27]. In 10% of our NRCD patients, further investigations, including duodenal biopsy, were normal and the symptom pattern was consistent with standard criteria for irritable bowel syndrome. It is possible that the original symptoms at presentation were functional and that the CD was an incidental diagnosis. Additionally, a GFD frequently fails to provide adequate fiber intake that may exacerbate constipation and symptoms of irritable bowel syndrome, for which we advise supplementary fiber with either an ispaghula or psyllium seed husk preparation. Clearly, the GFD should be continued if the diagnosis of CD has been confirmed. Patients with CD may also suffer from a range of other conditions that affect the general population and they should be investigated accordingly. It is not satisfactory to attribute any subsequent symptoms to a previous diagnosis of CD, particularly in cases in which symptoms initially responded to a GFD.

Responsive celiac disease

In our study of NRCD, nine patients were characterized as having RCD. Three were diagnosed with intestinal lymphoma, but one survived following treatment. At 2 years, 3/9 had died (33%), which is comparable to pre-existing cohorts[9,28]. There are no controlled trials but there are reports of symptomatic improvement with use of oral steroids and azathioprine. It is our practice to manage RCD and ulcerative jejunitis with moderate-dose prednisolone (20 mg/d), with initiation of azathioprine as a steroid-sparing agent (2-2.5 mg/kg). The steroid dose is tapered according to symptomatic response. We continue to monitor for the development of EATL. It is our practice to repeat duodenal biopsy after 4-6 mo to assess the small bowel inflammation and correlate this to ongoing symptoms and treatment.

Celiac serology

We test for serum IgA EMA and tTG antibodies in all patients with suspected CD, because these are the most sensitive and specific. We also test for IgA deficiency because this is over-represented in CD patients and can lead to a false-negative EMA result. We no longer recommend using anti-gliadin antibody testing because of poor specificity[19]. Initial reports have suggested that celiac serology is a good indicator of response to GFD[29]. However, a further study has indicated that serology correlates poorly with histological recovery[30]. In our experience of NRCD, there was a high rate of low titer positive serology and this disappointingly failed to correlate with specific causes. Although celiac antibody testing should be performed routinely in symptomatic CD, we do not feel that this should deter further investigation of the non-responsive patient.

Prognosis at two years

We have followed up this group of NRCD patients to provide information on longer-term outcome of NRCD. Only eight patients reported continued symptoms after 2 years, which included patients with RCD, as one might expect, and microscopic colitis. This is reassuring in that NRCD has a good prognosis if evaluated and managed appropriately.

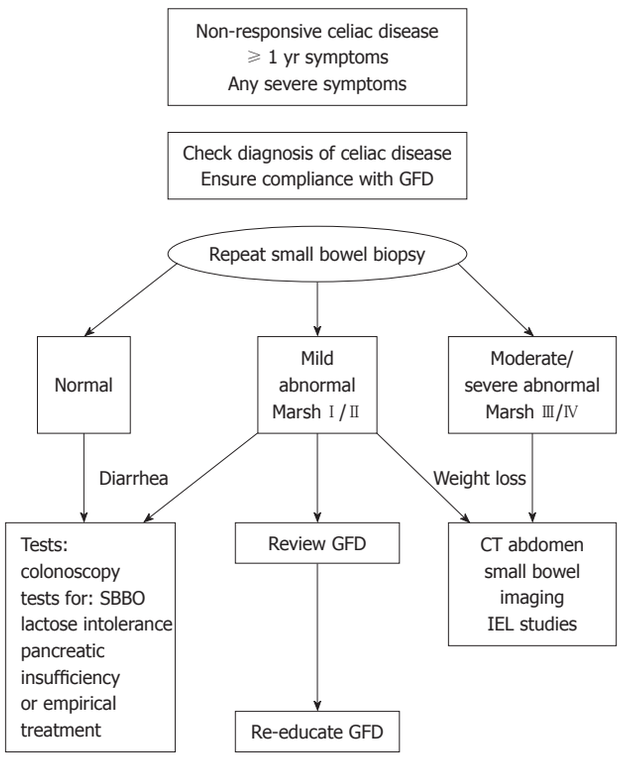

Figure 2 Algorithm for investigating non-responsive celiac disease. Moderate to severe abnormalities were defined by villous atrophy (Marsh Ⅲa-c, or Ⅳ)[20]. GFD: Gluten-free diet; SBBO: Small bowel bacterial overgrowth; IEL: Intraepithelial lymphocytes; CT: Computed tomography.

Investigation algorithm

This algorithm (Figure 2) has been used as a basic guide in the investigation of patients referred to our institution with NRCD. It reflects the pivotal role of repeat duodenal biopsy. It recognizes that mild histological abnormalities are more likely to indicate continued trace gluten intake or be present in the context of a secondary diagnosis. More severe histological changes or significant weight loss warrant more urgent investigation for RCD or intestinal lymphoma. In our study, all nine patients with RCD had significant weight loss and severe histological abnormalities on duodenal biopsy.

The management of NRCD depends on confirming the diagnosis of CD and establishing a cause for the symptoms, which should be possible in 90% of cases. We suggest that those with RCD should be evaluated for lymphoma and subsequently managed with immunosuppressive therapy. Alternative strategies involving treatment with cyclosporine[31], cladribine[32], or fluadribine and melaphan, stem cell transplantation for type Ⅱ RCD[33] have been reported, although their use is not generally accepted. Continued gluten ingestion accounts for 45% of persistent symptoms in patients with CD and a thorough and honest dietary assessment should be encouraged. Microscopic colitis and SBBO are impor-

tant causes of persistent ongoing symptoms that should respond to treatment. The longer-term prognosis of NRCD is good, with a 90% prospect of sustained symptom resolution.

COMMENTS

Background

Celiac disease (CD) is a common disease that affects approximately 1% of Northern Europeans and North Americans. It is an inflammatory condition predominantly involving the proximal small bowel in genetically susceptible individuals. Treatment involves a life-long gluten-free diet (GFD) with avoidance of dietary gluten present in wheat, rye and barley. Thirty percent of CD patients fail to improve or may relapse while on a GFD, which is termed non-responsive CD (NRCD).

Innovations and breakthroughs

The investigators report that the commonest cause of NRCD is continued gluten ingestion, either deliberately or by accidental ingestion. This cause is easily remediable by simple dietary measures. The authors also describe how other diagnoses can also contribute to the persistent symptoms: this includes microscopic colitis, a disease that causes diarrhea with a normal visual examination of the large bowel, and small bowel bacterial overgrowth; both of which occur more commonly in CD than previously reported.

Applications

This article helps investigation of NRCD through provision of an investigative algorithm for physicians to investigate the persistent symptoms in individuals with CD who have been prescribed a GFD. It also highlights that continued gluten intake and other diagnoses can be concomitant, such that they should be considered in the diagnostic work up.

Peer review

This is a good descriptive study in which authors investigate all patients referred to our centre with non-responsive celiac disease to establish a cause for their continued symptoms.The results are interesting and suggest that an algorithm for managing patients with non-responsive celiac disease.

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BRIEF ARTICLE

Second-line therapy for gemcitabine-pretreated advanced or metastatic pancreatic cancer

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Abstract

AIM: To investigate second-line chemotherapy in gemcitabine-pretreated patients with advanced or metastatic pancreatic cancer [(frequency, response, outcome, course of carbohydrate antigen 19-9 (CA 19-9)].

METHODS: This retrospective study included all patients with advanced or metastatic pancreatic cancer (adenocarcinoma or carcinoma) treated with second-line chemotherapy in our center between 2000 and 2008. All patients received first-line chemotherapy with gemcitabine, and prior surgery or radiotherapy was permitted. We analyzed each chemotherapy protocol for second-line treatment, the number of cycles and the type of combination used. The primary endpoint was overall survival. Secondary endpoints included progression-free survival, response rate, grade 3-4 toxicity, dosage modifications and CA 19-9 course.

RESULTS: A total of eighty patients (38%) underwent a second-line therapy among 206 patients who had initially received first-line treatment with a gemcitabine-based regimen. Median number of cycles was 4 (range: 1-12) and the median duration of treatment was 2.6 mo (range: 0.3-7.4). The overall disease control rate was 40.0%. The median overall survival and progression-free survival from the start of second-line therapy were 5.8 (95% CI: 4.1-6.6) and 3.4 mo (95% CI: 2.4-4.2), respectively. Toxicity was generally acceptable. Median overall survival of patients with a CA 19-9 level declining by more than 20% was 10.3 mo (95% CI: 4.5-11.6) *vs* 5.2 mo (95% CI: 4.0-6.4) for others (P = 0.008).

CONCLUSION: A large proportion of patients could benefit from second-line therapy, and CA 19-9 allows efficient treatment monitoring both in first and second-line chemotherapy.

Key words: Second-line; Chemotherapy; Pancreatic cancer; Gemcitabine; Carbohydrate antigen 19-9

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INTRODUCTION

Pancreatic cancer is the tenth most common cause of cancer in the United States and the fourth leading cause

of cancer death, with an estimated 42 000 new cases and 35 000 associated deaths in 2009[1]. In France, over 7200 patients were diagnosed with a pancreatic cancer in 2008, and almost the same number died from their disease[2]. At the time of diagnosis, most of patients present with advanced or metastatic pancreatic cancer, thereby precluding surgical resection[3]. Gemcitabine has been considered as the standard treatment for advanced pancreatic cancer ever since a randomized trial demonstrated significant improvement in survival and clinical benefit over 5-FU[4]. However, its efficacy remains moderate with median overall survival (OS) times ranging from 5 to 8 mo, and one-year survival rates varying between 17% and 25%. Numerous studies have attempted to increase efficacy of chemotherapy by combining gemcitabine with other drugs, but most of the regimens evaluated in phase Ⅲ trials failed to show any improvement in overall survival[5-18]. Only one randomized trial[6] (n = 569 patients) comparing gemcitabine alone *vs* gemcitabine combined with erlotinib showed a modest but significant increase in OS in the erlotinib arm (6.2 mo *vs* 5.9 mo, P = 0.025). Actually, the rate of patients receiving second-line chemotherapy varied from 16% to 57% in the trials evaluating a gemcitabine-based combination therapy[7-18]. This difference can be explained by both the deterioration in performance status after gemcitabine and the absence of recommended standard treatment in second-line[19]. Despite limited clinical data in this situation, a phase Ⅱ trial comparing oxaliplatin/folinic acid/5-FU (OFF) combination *vs* best supportive care as second-line treatment in gemcitabine-pretreated patients with advanced pancreatic cancer showed substantial benefit in the chemotherapy arm, with an overall survival prolonged by 2.6 mo (P = 0.008)[20].

Serum carbohydrate antigen 19-9 (CA 19-9), the sialyted Lewis blood group antigen defined by the monoclonal antibody 1116 NS 19-9[21], is the most common tumor marker in Europe and in the United States for patients with pancreatic cancer, both as a prognostic factor and an early marker of response to treatment. To date, the reliability and prognostic value of CA 19-9 levels to monitor first-line chemotherapy of advanced pancreatic cancer patients is well established[3].

In this context, this study aimed to describe the frequency of gemcitabine-pretreated patients with advanced or metastatic pancreatic cancer receiving second-line chemotherapy, their overall survival and progression-free survival. We also investigated response rates, outcome and potential correlations between the level and course of CA 19-9 and survival.

MATERIALS AND METHODS

Patients

This retrospective study included all adult patients with an advanced or metastatic histologically proven pancreatic cancer (adenocarcinoma or carcinoma) initially treated with gemcitabine in our center between 2000 and 2008. All patients received first-line chemotherapy with gemcitabine at a dose of 1000 mg/m^2 once weekly for 7 wk followed by 1 wk of rest; thereafter, gemcitabine was given once weekly for 3 wk followed by 1 wk of rest until progression of disease. Prior surgery or radiotherapy for local disease was permitted. All patients' medical records were registered within a computerized database [following national registry council (CNIL) authorization]. While there was no standard treatment used in second line, the treatment decision regarding a second-line therapy was systematically made by a multidisciplinary oncology committee according to the performance status, age and comorbidities.

Methods

We assessed each second-line chemotherapy protocol for the duration, the number of cycles and the type of drug combinations. The primary endpoint was OS. Secondary endpoints included progression-free survival (PFS), response rates, grade 3-4 toxicity, dosage modifications and CA 19-9 course. We stratified overall survival and progression-free survival according to the response to gemcitabine treatment (duration of treatment $\geqslant$ or $<$ 4 mo) and the performance status (0-1 *vs* 2-3). Response rates and disease progression were evaluated after 2 mo of treatment by Response Evaluation Criteria in Solid Tumors[22] and clinical examination. Toxicity was assessed at every visit using the National Cancer Institute Common Toxicity Criteria v.2.0 (CTC AE v2.0). The CA 19-9 levels were determined from serum samples collected at baseline (maximum one month before starting treatment) and at final treatment evaluation. A value of 60 IU/mL was accepted as the upper limit of normal. A reduction in CA 19-9 level was considered as relevant when serum concentrations decreased by more than 20% after the completion of treatment.

Statistical analysis

In this retrospective study, information relating to identification, treatment, available biological material, surgery, response to therapy and outcome were collected for each patient. The primary objective was to evaluate the efficacy of a variety of second-line regimens in a large series of advanced pancreatic adenocarcinoma after first-line treatment with a gemcitabine-based regimen. Categorical variables were reported by contingency tables. Continuous variables were expressed as medians and ranges. The objective response rate was presented with a 95% CI. Survival rates and median values were estimated according to the Kaplan-Meier method. Patients alive at the tie of analysis were censored at their last follow-up examination. Overall survival duration was measured from the date of first infusion until death from any cause. Progression-free survival duration was calculated from the date of first infusion until the first disease progression. Survival curves were drawn, and the log rank test was performed to assess differences between groups. All reported P values are two-sided. For all statistical tests, differences were considered as significant at the 5% level. Statistical analyses were performed using the STATA 9.0 software.

Table 1 Baseline patient characteristics in second line therapy

Clinical features	80 patients
Sex	
Male	38
Female	42
Median age (yr)	61.0
Histological diagnosis	67 (83.8)
OMS	
0	31 (38.7%)
1	40 (50.0%)
2	7 (8.8%)
3	2 (2.5%)
Presence of primary tumor	55 (68.8%)
Gemcitabine	
Median number of cycle	3.0 (1.0-12.0)
Median duration (mo)	3.3 (0.5-18.9)
Duration ⩾ 4 mo	29 (36.2%)
Metastatic disease	77 (96.3%)
Hepatic	70.1%
Peritoneal	29.9%
Nodal	23.4%
Pulmonary	16.9%
Carbohydrate antigen 19-9	
Initial median concentration (IU/mL)	741 (2.0-> 2000)
Elevated (> 60 IU/mL)	57 (89.1%)

Data are expressed as median values (range).

RESULTS

Patient characteristics

Baseline characteristics of the study population are detailed in Table 1. Of 206 patients receiving a first-line gemcitabine-based treatment for advanced or metastatic pancreatic cancer, 80 patients (38%) underwent a second-line therapy between January 2000 and May 2008. The median age was 61 years (range 36-81 years), and 38 patients were male (47.5%). The diagnosis of cancer was histologically confirmed in 67 patients (83.8%). Thirty-seven patients had undergone surgery including a pancreatoduodenectomy (n = 25) and palliative operation (n = 12) before first-line chemotherapy. Three other patients had received external radiation therapy. An endoscopic biliary prosthesis had been inserted prior to chemotherapy in eight patients. All patients received first-line chemotherapy with gemcitabine, with a median of 3 cycles (range: 1-12) and a median duration of 3.3 mo. Twenty-nine patients (36.2%) were treated for more than 4 mo. A total of 77 patients (96.3%) had evidence of metastatic disease, for most of them localized in the liver (70.1%). Despite the advanced stage of disease, patients generally showed good performance status before initiating second-line treatment, the WHO PS was of 0-1 in 71 patients (88.7%) and ⩾ 2 in nine patients (11.3%).

From the CA 19-9 analyses performed in 64 patients, fifty-seven (89.1%) showed an elevated level, and initial median serum concentration was 741.5 IU/mL (range: 2-2000 IU/mL).

Treatment

The median number of second-line chemotherapy cycles was 4 (range: 1-12) and the median duration of treatment was 2.6 mo (range: 0.3-7.4).

All treatment regimens are described in Table 2. Different drug combinations were used in second-line. Twenty-three patients (28.8%) received a treatment with cisplatin (cisplatin group), 22 patients (27.5%) with irinotecan (irinotecan group) and 21 patients (26.3%) with oxaliplatin (oxaliplatin group). Fourteen patients (17.5%) were given other treatment, including a single agent for four of them. The duration of treatment did not significantly differ between groups (Table 3).

Table 2 Treatment regimens in second line *n* (%)

Groups of chemotherapy	Patients
Cisplatin group	23 (28.8)
LV5FU2-CDDP	23 (28.8)
Irinotecan group	22 (27.5)
FOLFIRI	12 (15.0)
XELIRI	10 (12.5)
Oxaliplatin group	21 (26.2)
GEMOX	13 (16.2)
FOLFOX	8 (10.0)
Other group	14 (17.5)
5-FU alone	3 (3.7)
Gemcitabine + erlotinib	4 (5.0)
Gemcitabine + capecitabine	3 (3.7)
Capecitabine	1 (1.2)
5-FU + CDDP + RT	3 (3.7)

LV5FU2-CDDP: Folinic acid 400 mg/m², 5-FU bolus 400 mg/m², 5-FU 2400 mg/m² over 46 h and cisplatin 50 mg/m² on day 2, every 2 wk; FOLFIRI: Irinotecan 180 mg/m², folinic acid 400 mg/m², 5-FU bolus 400 mg/m², 5-FU 2400 mg/m² over 46 h, every 2 wk; XELIRI: Irinotecan 240 mg/m² and capecitabine po 2000 mg/m² J2-J15 every 3 wk; GEMOX: Gemcitabine 1000 mg/m² J1 and oxaliplatine 100 mg/m² J2, every two weeks; FOLFOX: Oxaliplatine 85 mg/m², folinic acid 400 mg/m², 5-FU bolus 400 mg/m², 5-FU 2400 mg/m² over 46 h, every 2 wk; 5-FU alone: 5-FU 250 mg/m² every day as continuous infusion; Gemcitabine + erlotinib: Gemcitabine 1000 mg/m² weekly X 7 for 8 wk then weekly X 3 out of 4 wk plus either erlotinib 100 mg po daily; Gemcitabine + capecitabine: Gemcitabine 1000 mg/m² weekly X 3 for 4 wk and capecitabine 1600 mg/m² J1-J21; Gapecitabine: 2500 mg/m² weekly X 2 for 3 wk; 5-FU + CDDP + RT: 60 Gy in 6 wk, 2 Gy/fraction, concomitant with 5-FU 300 mg/m^2 per 24 h as a continuous infusion, day 1-5 every week and cisplatin 20 mg/m^2 per day, day 1-5 at week 1 and 5.

Table 3 Chemotherapy regimens in second line

	Cisplatin group	Irinotecan group	Oxaliplatin group	Other group	*P* value
Number of patient	23 (28.8%)	22 (27.5%)	21 (26.3%)	14 (17.5%)	NS
Median number of cycle	5.0 (1.0-10.0)	5.0 (1.0-12.0)	4.0 (1.0-12.0)	2.0 (1.0-5.0)	NS
Median duration of treatment (mo)	2.7 (0.5-6.9)	3.2 (0.3-7.4)	2.3 (0.6-7.1)	2.3 (0.3-7.4)	NS
Disease control rate	10 (43.5%)	9 (40.9%)	9 (42.9%)	4 (28.6%)	NS
OS (mo)	6.7 (3.2-9.3)	4.5 (3.2-6.4)	4.5 (2.6-9.6)	5.2 (3.8-15.8)	NS
PFS (mo)	4.1 (1.9-6.7)	3.0 (2.0-6.1)	2.6 (1.8-5.4)	2.4 (2.1-10.1)	NS

OS: Overall survival; PFS: Progression-free survival; NS: Not significant.

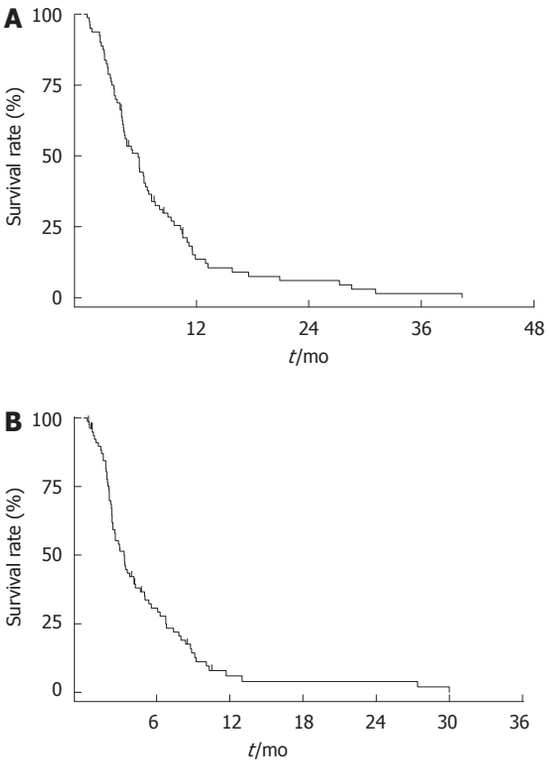

Figure 1 Survival from the start of second-line therapy. A: Overall survival; B: Progression-free survival.

Response and survival

There was no complete response. Six patients (7.5%) achieved a partial response, 26 patients (32.5%) a disease stabilisation, 44 patients (55.0%) experienced disease progression and 4 patients could not be assessed. The overall disease control rate (complete response, plus partial response, plus stable disease) was 40.0% (median follow-up was 6.0 mo).

The median OS from the start of second-line therapy was 5.8 mo (95% CI: 4.1-6.6 mo). The 1-year and 2-year OS rates were 13.6% (95% CI: 6.9-22.7 mo) and 6.1% (95% CI: 2.0-13.5 mo), respectively (Figure 1A). The median PFS from the start of second-line therapy was 3.4 mo (95% CI: 2.4-4.2 mo). The one-year and two-year PFS rates were 6.0% (95% CI: 1.8-13.9 mo) and 4.0% (95% CI: 0.8-11.5 mo), respectively (Figure 1B). There was no significant difference between the four chemotherapy groups for overall disease control rates, overall survival and progression-free survival ($P > 0.05$) (Table 3).

The median OS was 6.3 mo (95% CI: 4.3-7.2 mo) in patients with a performance status of 0-1 (71 patients) *vs* 1.8 mo (95% CI: 0.3-5.9 mo) in patients with a PS > 1 (9 patients) ($P < 0.001$). The one-year OS rates were 16.0% and 0%, respectively. The median PFS was 3.4 mo (95% CI: 2.6-4.9 mo) in patients with a performance status of 0-1 *vs* 2.1 mo (95% CI: 0.5-3.0 mo) in patients with a PS > 1 ($P = 0.004$). The one-year PFS rates were 7.0% and 0%, respectively.

The median OS times were 7.2 mo (95% CI: 4.5-10.5 mo) in patients treated for more than 4 mo with gemcitabine as first-line therapy (29 patients) and 4.2 mo (95% CI: 3.2-5.9 mo) in those treated less than 4 mo (51 patients) ($P = 0.046$). The one-year PFS rates were 10.0% and 4.0%, respectively.

Toxicity and dosage modifications

Toxicity was generally acceptable. The incidence of severe adverse events (grade 3-4) is reported on Table 4. Twenty-seven patients (33.7%) experienced at least one grade 3-4 toxic event. Neutropenia was the most frequent haematological toxicity, occurring in 14 patients (17.1%). There were 5 chemotherapy-related deaths. Two deaths were attributed to sepsis, and three to a combination of cancer and treatment-related complications. There was no difference in the incidence of toxicity and treatment-related deaths between the four chemotherapy groups (Table 5).

Forty-one patients (51.3%) had dosage modifications, including treatment suppression for 7 patients, dose reduction for 17 patients and cycle delay for 33 patients. Dose reductions were caused by haematological (9 patients, 53%) or clinical toxicities (8 patients, 47%) (Table 6). In thirty-one patients (41.3%), the chemotherapy was discontinued before evaluation because of disease progression (74.2%), toxicity (9.7%) or death (16.1%). There was no significant difference between groups for dose modification and chemotherapy discontinuation before evaluation.

Carbohydrate antigen 19-9 measurement and survival

Reduction in CA 19-9 levels during treatment was associated with improved survival. The median OS was significantly higher in patients whose level of CA 19-9 declined by more than 20% when compared to other patients 10.3 mo (95% CI: 4.5-11.6) *vs* 5.2 mo (95% CI: 4.0-6.4) ($P = 0.008$) (Figure 2A). In this subgroup of patients, the median PFS was 6.7 mo (95% CI: 3.3-8.8 mo) *vs* 3.4 mo (95% CI: 2.6-4.2 mo) ($P = 0.031$) (Figure 2B). All patients who experienced a CA 19-9 reduction > 20% achieved disease control (3 partial responses and 5 cases of stable disease).

DISCUSSION

If gemcitabine-based chemotherapy is the current standard of care for first-line treatment of advanced pancreatic cancer, there are limited data to support a standard second-line chemotherapy regimen[23]. Indeed, the true survival benefit from first-line therapy is small, and few patients can endure a second line as their performance status deteriorates with disease progression. In our study, the rate of patients treated with second-line chemotherapy was 38.8%, in accordance with most published data regarding gemcitabine-pretreated pancreatic cancer (16%-57%). Median overall survival from the start of second-line setting was 5.8 mo (4.1-6.6 mo), and median progression-free survival was 3.4 mo (2.4-4.2 mo). These results are similar to those obtained in first-line with gemcitabine by Burris *et al*[4] or Heinemann *et al*[9].

Table 4 Toxicity, dosage modifications and chemotherapy discontinuation *n* (%)

	Patients
Clinical toxicity grade 3-4	
Nausea	3 (3.7)
Vomiting	5 (6.2)
Diarrhea	2 (2.4)
Stomatitis	1 (1.2)
Fever	6 (7.5)
Infection	6 (7.5)
Haematological toxicity grade 3-4	
Anemia	2 (2.4)
Neutropenia	14 (17.1)
Thrombocytopenia	1 (1.2)
Dosage modifications	41 (51.3)
Type	
Treatment suppression	7 (17.1)
Dose reduction	17 (41.5)
Delay of cycle	33 (80.5)
Discontinuation before evaluation	31 (41.3)
Progressive disease	23 (74.2)
Toxicity	3 (9.7)
Chemotherapy-related deaths	5 (16.1)

Table 5 Toxicity for chemotherapy groups *n* (%)

	Cisplatin group	Irinotecan group	Oxaliplatin group	Other group	*P* value
Clinical toxicity grade 3-4					
Nausea	0	0	3 (14.3)	0	NS
Vomiting	2 (9.1)	0	2 (9.5)	1 (5.3)	NS
Diarrhea	1 (4.5)	1 (5.6)	0	0	NS
Fever	0	4 (22.3)	1 (4.8)	1 (5.3)	NS
Infection	0	3 (16.7)	3 (14.3)	0	NS
Haematological toxicity grade 3-4					
Anemia	0	1 (5.6)	1 (4.8)	0	NS
Neutropenia	6 (27.3)	6 (33.4)	2 (9.5)	0	NS
Thrombocytopenia	1 (4.5)	0	0	0	NS

NS: Not significant.

Table 6 Dose reduction *n* (%)

Dose reduction	Patients
Neutropenia grade 2 or 3-4	5 (6.2)
Thrombocytopenia grade 2	4 (5.0)
Hand-foot skin reaction grade 2	5 (6.2)
Neuropathy grade 2	2 (2.4)
Diarrhea grade 3-4	1 (1.2)

Moreover, patients with good performance status (0-1) and who had benefited from gemcitabine chemotherapy in first line (duration of treatment ⩾ 4 mo) had a significantly greater duration of overall survival than those who had not (6.3 mo *vs* 1.8 mo, $P < 0.001$; and 7.2 mo *vs* 4.2 mo, $P = 0.046$, respectively). The rate of grade 3-4 toxicity was determined to be 33.7% (27 patients), but there were no unexpected side effects. Consequently, our experience demonstrates that a selected population of

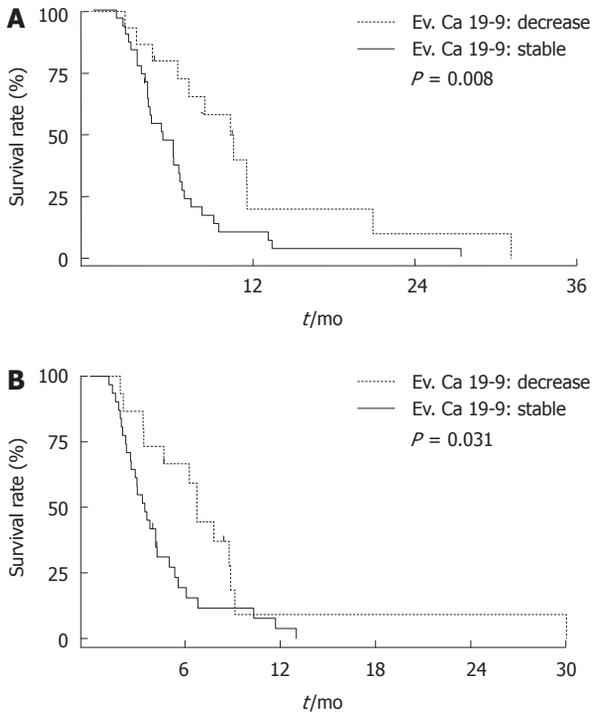

Figure 2 Overall survival and carbohydrate antigen 19-9 evolution in second line. A: Overall surviva; B: Progression-free survival. Ev. Ca 19-9: Course of carbohydrate antigen 19-9.

patients with good performance status can benefit from second-line chemotherapy after first-line gemcitabine-based treatment, with appreciable overall and progression-free survivals. This retrospective study included a large population, while most of data published over the last ten years involved relatively small samples in monotherapy (from 13 to 52 patients) as well as in bitherapy (from 12 to 46 patients)[24]. The disease control rate was 40%, as described by many authors for both monotherapy and bitherapy regimens, and median overall and progression-free survivals were superior to those reported in monotherapy studies, but were not different from bitherapy[24].

In daily practice, second-line therapies are regularly used in gemcitabine-pretreated patients with pancreatic carcinomas, but the efficacy and benefit in terms of survival or quality of life have never been validated. A randomized phase Ⅲ trial conducted in second line was presented by Pelzer *et al*[25]. One hundred and sixty-five gemcitabine-pretreated patients with pancreatic cancer were randomly assigned to receive either FF (5-FU 2 g/m^2 for 24 h plus folinic acid or leucovorin 200 mg/m^2 on days 1, 8, 15 and 22) or OFF (FF plus oxaliplatin 85 mg/m^2 on days 8 and 22). Median overall survival and progression-free survival were significantly improved with OFF protocol (20 wk *vs* 13 wk, $P = 0.014$; and 13 wk *vs* 9 wk, $P = 0.012$, respectively), with an acceptable tolerance profile. This study illustrated the effectiveness of this protocol which may become the standard second-line therapy. Currently, the National Comprehensive Cancer Center pancreatic cancer guidelines encourage the participation of patients with satisfactory performance status in

clinical trials, and recommend the use of oxaliplatin and fluoropyrimidine if enrolment in trials is not possible[26,27]. Finally, the XELOX regimen[28] showed comparable efficacy to FOLFOX (or OFF) regimen, while offering the advantage of oral fluoropyrimidine treatment. Even so, more large randomized controlled trials are required in second line before a new standard of care can be established.

Interestingly, the CA 19-9 measurement was correlated with OS and PFS in our study. Patients whose level of CA 19-9 declined by more than 20% had a significantly greater duration of survival. The prognostic value of CA 19-9 level and course is well established for patients with pancreatic cancer treated with surgery[29-31], radiotherapy and chemoradiotherapy[32,33]. Some studies also correlated the level and the course of CA 19-9 with OS and PFS of pancreatic cancer patients treated with gemcitabine as first-line chemotherapy[3,34-36]. These studies showed improved median OS for patients with a decrease of CA 19-9 > 20% after two months of treatment with gemcitabine. Saad *et al*[37] reported an increase in the median OS for patients with a reduction of CA 19-9 at any time after treatment. In second-line, only one study demonstrated that a CA 19-9 value > 400 IU/mL was a significant independently negative prognostic factor[38]. To our knowledge, it was the second report which showed a correlation between OS and CA 19-9 course[39], and the first report for PFS and CA 19-9 course in second-line chemotherapy for gemcitabine-pretreated patients with pancreatic cancer.

In summary, treatment of metastatic pancreatic cancer remains a major challenge and requires new chemotherapeutic and targeted agent combination to be compared to gemcitabine in first-line. It should be noted that a new therapeutic alternative could merge in first-line for selected patients according to the recent results obtained in a randomized Phase Ⅲ study comparing FOLFIRINOX regimen to gemcitabine[40]. A significant longer overall survival, progression-free survival, and higher response rates were obtained with FOLFIRINOX than with gemcitabine alone, associated with manageable toxicities.

The present study focused on second-line therapy in gemcitabine-pretreated patients with advanced pancreatic cancer. From our experience, second-line chemotherapy is a valuable treatment option after progression on gemcitabine-based regimen, because 30% to 40% of patients could benefit from this therapy, especially those with good performance status (1-2) and who gained benefit from first-line therapy. Further randomized clinical trials are necessary to provide a standard treatment in this situation. Additionally, measurement of the CA 19-9 level was confirmed to be an efficient marker for treatment monitoring in first-line as well as in second-line treatment.

COMMENTS

Background

Most of patients have advanced or metastatic pancreatic cancer at the time of diagnosis, and cannot benefit from surgery. Gemcitabine-based chemotherapy is the standard treatment in first-line, but there are limited data to support standard second-line chemotherapy.

Research frontiers

In practice, second-line therapies are regularly used in gemcitabine-pretreated pancreatic carcinomas, but the efficacy and benefit in terms of survival or quality of life have never been validated. Most of published studies in second line involved small samples, in monotherapy as well as in bitherapy.

Innovations and breakthroughs

In our study, the rate of patients treated with second-line chemotherapy was 38.8%, and median overall and progression-free survivals from the start of second-line were similar to those obtained in first-line with gemcitabine. Carbohydrate antigen 19-9 (CA 19-9) course was correlated with prolonged overall and progression-free survival.

Applications

Second-line chemotherapy is a valuable treatment option after progression on gemcitabine-based regimen, because 30% to 40% of patients could benefit from this therapy. Measurement of the CA 19-9 level was confirmed to be an efficient marker for treatment monitoring, in first-line as well as in second-line treatment.

Peer review

The study is very interesting because there is no consensus about second-line therapy after disease progression while patients are receiving gemcitabine. The paper is well written. However, the authors should revise several points in the entire text.

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BRIEF ARTICLE

Post-cholecystectomy symptoms were caused by persistence of a functional gastrointestinal disorder

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Abstract

AIM: To classify gallstone disease as a basis for assessment of post-cholecystectomy symptoms.

METHODS: One hundred and fifty three patients with a clinical and ultrasonographic diagnosis of gallstones filled out a structured questionnaire on abdominal pain symptoms and functional gastrointestinal disorder (FGID) before and at six months after cholecystectomy. Symptom frequency groups (SFG) were categorized according to frequency of pain attacks. According to certain pain characteristics in gallstone patients, a gallstone symptom score was accorded on a scale from one to ten. A visual analogue scale was used to quantify pain. Operative specimens were examined for size and magnitude of stone contents as well as presence of bacteria. Follow-up took place after six months with either a consultation or via a mailed questionnaire. Results were compared with those obtained pre-operatively to describe and analyze symptomatic outcome.

RESULTS: SFG groups were categorized as severe (24.2%), moderate (38.6%) and mild (22.2%) attack frequency, and a chronic pain condition (15%). Pain was cured or improved in about 90% of patients and two-thirds of patients obtained complete symptom relief. Patients with the most frequent pain episodes were less likely to obtain symptom relief. FGID was present in 88% of patients pre-operatively and in 57% post-operatively (P = 0.244). Those that became asymptomatic or improved with regard to pain also had most relief from FGID (P = 0.001). No pre-operative FGID meant almost complete cure.

CONCLUSION: Only one third of patients with FGID experienced postoperative relief, indicating that FGID was a dominant cause of post-cholecystectomy symptoms.

Key words: Gallstone symptoms; Functional gastrointestinal disease; Cholecystectomy; Post-cholecystectomy symptoms

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INTRODUCTION

It is commonly accepted that removal of the gallbladder is the best treatment for symptomatic gallstone disease. However, less focus has been on patient selection and typical or common symptoms of this disease in order to understand prevailing symptoms after surgery[1-4]. Although disease severity has been used[5-7], these efforts have not been united into useful and widely accepted working terms for preoperative clinical use and outcome assessment. As a consequence, the indication for cholecystectomy is sometimes vague and assessment of outcome suffers accordingly[8].

Pain is a key element in gallstone symptoms but pain is a general symptom. Therefore pain characteristics and additional symptoms reported in classical descriptions of the disease has expanded the interpretation[9-11]. Functional gastrointestinal disorder (FGID) is quite common in the population and the two diseases often appear together[12]. FGID may go away or appear more distinct to the patient after cholecystectomy and thus distort the sense of postoperative relief. Up to 30% of patients have some symptoms following cholecystectomy[13,14]. No consistent physiological substrate for such pain has been documented[2]. It is somewhat unclear to what degree post-cholecystectomy symptoms resemble the exact symptoms before removal of the gallbladder[13]. Most studies are retrospective with follow-up periods commonly ranging from a few weeks to a couple of years[6,14,15]. A recent, prospective study lacked clinically useful working terms with a mixture of both pain associated symptoms and FGID[3].

Our aim was to categorize gallstone disease according to the severity of clinical symptoms, pain characteristics, and quantify FGID to define postoperative outcome in terms of new or persistent symptoms.

MATERIALS AND METHODS

One hundred and fifty-three patients with an ultrasonographic diagnosis of gallstones admitted for elective laparoscopic treatment of symptomatic, uncomplicated gallstone disease in a Norwegian (n = 100) and a US (n = 53) institution.

Questionnaire

The patients filled out a structured questionnaire on pain characteristics and functional abdominal symptoms before and at six months after surgery. The questionnaire was assembled after a large experience with pre-operative interviews in two randomized trials and modeled as a simplified version of the Rome Ⅱ questionnaire[16,17]. Symptoms were classified according to appearance ranging from never to almost always in four steps: never, occasionally, very often, and almost always. Only those symptoms that were present more than 50% of the time (i.e. the last two) was counted as a positive answer (Table 1).

Follow-up was conducted at the outpatient clinic for all Norwegian patients at six months at which time the questionnaire was filled out. The American patients were mailed the questionnaire for logistic reasons.

Gallstone pain attacks were categorized as symptom frequency groups (SFG) according to the frequency experienced during the last three months. Patients that were unable to define exact time periods for pain attacks or had a dominant pattern of ubiquitous pain or had symptoms dominated by severe nausea or food intolerance were classified as a chronic symptom group.

A visual analogue scale score (VAS) was used to quantify the severity of pain in the symptom questionnaire. A 100 mm long horizontal line was to be marked vertically at the point consistent with the pain experienced by the patient. The left end was marked "No pain" and the right end "Unbearable pain".

Gallstone symptom score

According to certain pain characteristics in gallstone patients, a Gallstone symptom score (GSS) was accorded from 1 to 10 (Tables 2 and 3).

In our practice, patients were found to have symptomatic, uncomplicated gallstone disease if ultrasonography detected gallbladder stones and the patients had relevant clinical symptoms. Endoscopy was neither a routine part of the pre-operative work-up nor planned as a diagnostic aid in case of persistent symptoms.

Pathology

Operative specimens were prepared for examination of bacterial contents, stone size and routine histology on the back table immediately after the operation finished.

Bile was aspirated with a syringe from the gallbladder and sent for culture together with a piece of the wall. Stone size was measured with a caliper after the gallbladder had been opened on the back table. Finally the specimen was put on formalin and sent to the pathologist for routine (hematoxylin and eosin) staining and histological assessment.

Ethics

The Regional Ethical Committee of Western Norway and The National Data Inspectorate approved the study. The Institutional Review Board (IRB) of Cleveland Clinic approved the study (IRB 7000/04). The study was registered with clinicaltrials.gov as part of NCT01190280.

Table 1 Assessment of functional abdominal symptoms (functional gastrointestinal disorder)

Perspiration
Intolerance to food
Acid regurgitation
Heartburn
Difficulty swallowing, food sticking in the lower esophagus
Nausea
Loss of appetite (anorexia)
Feeling full after eating very little (early satiety)
Feeling of abdominal fullness or bloating
Abdominal distension, which requires loosening of the belt
Frequent loose bowel movements (or more often than usual)
Constipation (or less bowel movements than usual)
Alternating constipation and loose bowel movements
Difficulty passing stools with straining, urgency or feeling of incomplete evacuation
Abdominal pain or discomfort is relieved by bowel movements (passing of stool)

Rate the frequency of the following symptoms associated with abdominal pain during the last 3 mo or longer, using the following scale: 0: Not at all or rarely (less than 10% of the episodes); 1: Occasionally (less than 50% of the episodes); 2: Very often (more than 50% of the episodes); 3: Almost always (more than 80% of the episodes).

Statistical analysis

The χ^2 test was used to compare the level of improvement between groups, and to compare the presence of FGID between patients with different symptom alleviation before and after operation. Logistic regression for dependent paired data was used to analyze the difference in FGID before and after surgery between different GSS-groups. The statistical software used was PASW Statistics version 18.0 and Intercooled Stata 9.2 for Macintosh.

RESULTS

The patient demographics are shown in Table 4.

Symptom frequency groups and visual analogue scale score

Four SFG were categorized according to frequency of pain attacks: severe (24.2%): ⩾ 1 pain attack per week, moderate (38.6%): ⩽ 3 pain attacks per month, mild (22.2% of the patients): ⩽ 2 pain attacks per 3 months, or chronic pain condition (15%): no discernable pain attack pattern.

The VAS was equally distributed between all patients, mean VAS preoperatively was 82.8 with variation from 17 to 100 (Table 5).

Gallstone symptom score

Mean preoperative GSS in pair-wise comparisons showed a significant difference preoperative between chronic and moderate disease patients ($P = 0.022$). There was a non-significant trend towards a greater rate of cure or symptom relief measured with GSS among patients with less severe disease ($P = 0.651$). Patients in the most severe SFG had the highest GSS and experienced more remaining symptoms, for details see Tables 5 and 6.

Table 2 Assessment of pain symptoms

Had an abdominal pain attack at least once for the last 3 mo or longer?
Experienced either pain or discomfort in the abdomen of a continuous steady nature at least once per week for the last 3 mo or longer?
For women: Did the onset of pain begin during pregnancy or soon after pregnancy?
Evaluated in the Emergency Department or seek medical attention for the abdominal pain?
Admitted to the hospital for the abdominal pain?
Estimate how often pain medications are required for the pain:
Not at all or rarely (less than 10% of the episodes)
Occasionally (less than 50% o the episodes)
Very often (more than 50% of the episodes)
Almost always (more than 80% of the episodes)
Time-interval during which the pain most often occurs:
7 am – 12 pm
12 pm – 6 pm
6 pm – 11 pm
11 pm – 7 am
Highly variable and unable to predict time of onset
Rate how often the pain occurs in the following abdominal areas:
Right upper quadrant[1]
Left upper quadrant[1]
Right lower quadrant[1]
Left lower quadrant[1]
Midline or center of the upper abdomen[1]
Is there often an area where the pain is strongest (able to point with one or two fingers):
Right upper quadrant
Left upper quadrant
Right lower quadrant
Left lower quadrant
Midline or center of the upper abdomen
Highly variable and unable to predict one area
No
Experience discomfort in the right upper quadrant when bending forward?
Abdominal pain radiates from where it started?
If yes, where does it radiate most often?
Right upper back beneath the right shoulder blade
Upper back between the shoulder blades
Lower back
None of these places mentioned
Highly variable and unable to predict a dominant area
Estimate the number of pain attacks over the last 3 mo
Estimate the usual duration of a pain attack in hours and minutes
Experience urge to move around during a pain attack[1]
Choose one of four patterns describing pain attacks (depicted by graphs):
Low-grade warning pain followed by a steady rise to a maximal constant pain, gradually getting better after a while
Low-grade warning pain followed by a steady rise to a maximal degree with occasional waves of pain, gradually getting better after a while
Pain begins suddenly with maximal intensity and improves over time
Pain begins suddenly with maximal intensity and persists with waves of pain until it goes away
Rate level of maximal pain intensity by 100 mm visual analogue scale score-scale

Pain attacks are defined as suddenly appearing pain that is distinct from, and stronger than any continuous, steady pain or discomfort. [1]The pain occurrence in each area is rated as: not at all or rarely (less than 10% of the episodes), occasionally (less than 50% o the episodes), very often (more than 50% of the episodes) or almost always (more than 80% of the episodes).

Functional gastrointestinal disorder symptoms

A FGID was present in 87.6% before surgery and in 57.6%

Table 3 Assignment of a clinical gallstone symptom score to different preoperative symptom frequency groups (%)

Symptom	Score	Percent of patients with symptoms according to pain presentation			
		Severe	Moderate	Mild	Chronic
Pain in upper abdomen: Pain most common in right upper quadrant or intensifies when bending forward or lying on the right side	2	100	96.6	94.1	88.2
Pain attacks commonly last more than one hour	1	73.0	66.7	76.5	46.2
Pain in a "plateau fashion"	1	62.2	72.9	67.6	64.3
Urge to move during pain attacks	1	51.4	69.0	58.8	84.6
Pain commonly occurs at night	1	43.2	61.0	50.0	29.4
Pain radiation to the back	1	40.5	47.5	38.2	58.8
Nausea during pain attacks	1	61.1	48.3	52.9	50.0
Use of analgesics in > 50% of pain attacks	1	54.0	54.3	44.1	41.2
Perspiration during pain attacks	1	36.1	41.4	41.2	60.0

Table 4 Demographics of the study population of 153 patients and 115 follow-up responders *n* (%), mean age (range, yr)

Symptom frequency group	Females	Males	Total
All groups	122 (79.7), 47 (17-81)	31 (20.3), 51 (28-85)	153 (100), 48 (17-85)
Severe disease	31, 45 (17-81)	6, 44 (25-64)	37 (24.2), 45 (17-81)
Moderate disease	47, 44 (20-72)	12, 53 (39-70)	59 (38.6), 46 (20-72)
Mild disease	26, 53 (25-78)	8, 52 (34-85)	34 (22.2), 53 (25-85)
Chronic disease	18, 53 (23-81)	5, 55 (30-80)	23 (15.0), 54 (23-81)
Responders to follow-up	89 (77.4), 49 (20-81)	26 (22.6), 52 (25-85)	115 (75.2), 50 (20-85)

χ^2 for gender; $P = 0.889$.

Table 5 Changes in gallstone severity score by symptom frequency group in 115 responding patients from the study population of 153 patients *n* (%)

Preoperative SFG	Patients	Preoperative		Responders	Postoperative		mean % reduction in GSS
		mean GSS	mean VAS		mean GSS	mean VAS	
Severe disease	37 (24.2)	6.11	81.1	29 (78.4)	1.76	33.0	69.1
Moderate disease	59 (38.6)	6.47	86.6	41 (69.5)	1.32	15.8	78.7
Mild disease	34 (22.2)	6.09	81.3	26 (76.5)	1.04	12.8	87.0
Chronic disease	23 (15.0)	4.35	76.8	19 (82.6)	1.00	8.9	62.7

SFG: Symptom frequency groups; GSS: Gallstone symptom score; VAS: Visual analogue scale score.

at follow-up after surgery. No difference was seen between the different SFG ($P = 0.244$). There was a trend that patients with FGID before surgery were less likely to experience improvement of their pain or complete relief. Likewise, patients without FGID after surgery were more likely to report improvement or complete relief of pain (Table 7).

Gallbladder specimen examination

Histology of the gallbladder showed that 85% had chronic and 10% subacute inflammation while 5% were normal. Bacteriological examination in 79 patients discovered bacteria in 12 (15.2%) without difference between the groups. The distribution of bacteria was: gram-negatives 3.8%, gram-positive cocci 8.9%, and mixed cultures 2.5%. Stone type was not examined.

The number of stones was measured in 66 patients and size in 64 patients. The mean number was 2.5 (range 1-9) with variation between SFG from 2.3 to 2.8. The stone size was mean 13 mm (range 1-40) with variation between groups from 12.5 to 13.2 mm. There were no statistically significant differences between the groups.

DISCUSSION

Gallstone symptoms are still classified simply as biliary colic long after a variety of pain characteristics have been described for these pain attacks[9,10]. Thus, studies of outcome of gallstone disease are usually hampered by lack of scientifically acceptable definitions and designs[3,5,6,16]. This includes an inadequate definition of gallstone symptoms, lack of proper recognition of FGID as a concomitant complaint, prospective design and defined follow-up methods. Freedom of pain attacks is a major outcome measure after cholecystectomy. Complete cure of a biliary type pain in contrast to a persisting dull aching pain, has been reported as a reasonable goal for surgery[18]. Previous studies have reported that so-called

Table 6 Symptomatic improvement in 115 patients after cholecystectomy *n* (%)

	Groups			
	Asymptomatic but improved	Symptomatic or worse	Unchanged	*P* value[1]
Patients				0.651
All patients	76 (66.1)	28 (24.3)	11 (9.6)	
Severe disease	15 (51.7)	10 (34.5)	4 (13.7)	
Moderate disease	27 (65.8)	9 (22.0)	5 (12.2)	
Mild disease	20 (76.9)	5 (19.2)	1 (3.9)	
Chronic disease	14 (73.7)	4 (21.0)	1 (5.3)	
Age				0.490
< 60	54 (64.3)	23 (27.4)	7 (8.4)	
> 60	22 (71.0)	5 (16.1)	4 (12.9)	
Gender				0.573
Women	56 (62.9)	24 (27.0)	9 (10.1)	
Men	20 (76.9)	4 (15.4)	2 (7.7)	

[1]*P* values from χ^2 calculation.

Table 7 Presence of pre- and post-operative functional gastrointestinal disorder in 115 patients with different *n* (%)

Presence of FGID	Patients	Asymptomatic	Symptomatic, improved	Unchanged or worse	*P* value[1]
None pre-operative	13 (11.3)	11 (84.6)	2 (15.4)	0	
Present pre-operative	102 (88.7)	65 (63.7)	26 (25.5)	11 (10.8)	0.449
None post-operative	49 (42.6)	42 (85.7)	7 (14.3)	0	
Present post-operative	66 (57.4)	34 (51.5)	21 (31.8)	11 (16.7)	0.001
Total	115 (100)	76 (66.1)	28 (24.3)	11 (9.6)	

FGID: Functional gastrointestinal disorder.[1]*P* values from χ^2 calculation.

biliary colic remained in only 8%-9% of patients in contrast to non-colicky pain in 18%-32%[13,19]. Others have found an incidence of around 20% of persistent pain of the same character as before the operation[20,21]. Lublin and coworkers[6] reported presence of pain in 25% and non-pain symptoms in 43% . It seems that distinct or marked pain is present in up to 4%-9%[12,13] whereas pain or "discomfort" connected with dyspeptic symptoms are not clearly categorized[22]. Around 25% of our patients improved without being completely cured after removal of the gallbladder. This corresponds to previous figures of 18%[13] and the frequency of more diffuse intestinal symptoms found by others during post-operative examination[12]. One author mentioned similar findings without giving figures but did not find interference with quality of life measurements[23]. Up to 93% satisfaction has been reported after removal of the gallbladder[13,15,22,24,25].

FGID consists of two main subgroups, functional dyspepsia and irritable bowel syndrome (IBS), with overlapping features making them both symptomatic of an irritable or dysfunctional gut[26,27]. The criteria in the Rome Ⅱ and the more recent Rome Ⅲ questionnaire give a formal definition of FGID[17,28]. In the West, there tends to be a female predominance. FGID appears as a real condition of gallstone disease[3,12,22,29]. The pathological connection is still obscure but a common dysfunctional trait has been shown[30]. A diagnostic problem arises only when gallstone disease becomes vague with regard to pain expression[3,5,22]. Lublin and coworkers[6] reported that 80% of patients had so-called non-pain symptoms pre-operatively in accordance with an 88% incidence of FGID in our patients. In our practices, nearly all gallstone patients coming to surgery have upper abdominal pain either in the right upper quadrant or epigastrium although a small percentage has intolerable nausea or food intolerance that dominates over pain. FGID was therefore judged a concomitant condition in most cases. Our outcomes are quite similar to those of others who have attempted to classify pre-operative symptoms[5,6]. It could be perceived that freedom of pain with an attack pattern was the decisive factor when cure or relief was achieved, whereas FGID of various intensities caused the bulk of the persistent symptoms, because FGID persisted in 57% of the patients. The post-operative GSS and VAS were markedly decreased and it is therefore likely that the patients were cured of the pain attacks that led to cholecystectomy. Besides, even so-called bilary colic, even if it resembles pre-operative symptoms, needs a substrate when the gallbladder has been removed. It has not been proved that this stems from the common bile duct (CBD) or the sphincter of Oddi, even though symptoms caused by CBD disease, such as a stone, may be quite similar. Therefore, we will argue that there is reasonable evidence pointing to FGID as a cause of persisting symptoms after surgery.

Some investigators have reported that patients with the most severe, frequent or bothersome pre-operative symptoms are less likely to be cured[5,6,13,22]. The present study corroborated this as results showed that only frequency of pain attacks expressed as SFG separated the patients with regard to severity in the pre-operative evalu-

ation. GSS only separated the pain attack groups against the chronic group. This is broadly correlated with a Swedish study but differed insofar that we amalgamated what were their two most severe groups into one[5]. Lublin and coworkers[6] used frequency without a more specific definition. The disease may wax and wane and this may influence the response to the questionnaire[3,5]. A minority of 15% had chronic symptoms with daily occurrence as the rule. We suspect that some of the patients with daily symptoms reported by Halldestam and coworkers[5] might have been classified as a chronic symptom group by our definition. This would distort comparison of outcome because these two groups responded differently to operative treatment in our study. It is also difficult to ascertain the meaning of "atypical" or multiple locations of pain[5]. Pain in the right upper quadrant or epigastrium is a core issue in the diagnosis of gallstone disease but admittedly in a small minority of patients other symptoms dominate. However, as long as these symptoms can be assigned to gallstone disease, they are not a contraindication to surgery in such cases.

Although patients with the highest pre-operative mean GSS had the largest relative score reduction, this group retained a higher post-operative score and had the highest VAS score. The reason for that was largely assumed as being caused by persistent symptoms of FGID even though this could not be established with certainty because of overlapping symptoms in gallstone patients. It was, however, consistent with the observation that the severe SFG had more patients with no relief and also had a slightly larger GSS burden and consequently higher post-operative GSS and VAS score, indicating that a larger disease burden or more symptoms was in concert with a higher VAS. This may be interpreted as more persistent pain. One study found that patients with the most bothersome symptoms before surgery had less chance of cure[22]. The highest odds ratio for persistence was obtained by "gas/flatulence", a common symptom of IBS or FGID. This could easily be interpreted as caused by FGID but it has been unusual to explicitly label post-cholecystectomy symptoms as FGID even though many symptoms fit this diagnosis[22]. One explanation may be that these symptoms has for too long been discerned as part of a wider range of gallstone symptoms while we will argue that they are two concomitant disease expressions with many overlapping features making it difficult to separate them.

Compared with measurements before surgery VAS has reached levels of around 68 (of 100) pre-operatively to levels of 35 to 45 post-operatively[13,15,19,21]. In the present study, VAS was similar across all four GSS groups and it fell after surgery to a mean of 18 (range 9 to 33). Therefore, it could not by itself be used to distinguish between the patients before or after surgery. Our post-operative median score value indicates no more than mild to moderate pain[31].

Theoretically, a bile duct focus might cause pain quite similar to that originating in the gallbladder but only about 2% has common bile duct stones after removal of the gallbladder[6,13]. Psychometric testing has shown that a psychosomatic disturbance may influence outcome after cholecystectomy[8,32]. It has been observed that women tend to have more postoperative pain[33] while some have reported that gender is irrelevant[15,20,34]. Women under the age of 60-years have been found to have significantly more pain of the diffuse, more continuous type that is also described in functional dyspepsia, and satisfaction has been greater for men[13]. We found that women were less likely to become asymptomatic. Age of the patient has not influenced outcome[20,34], whereas the opposite was found when 50 or 55-years-of-age was used as cut off value[4,22]. In contrast to previous studies, patients more than 60-years-of-age fared slightly worse in the present study[5,13]. Stone size and number, bacteriology, or histology[2] did not impact the symptom presentation in this study.

We recommend a follow-up period of 6 mo before assessing outcome after cholecystectomy[8,22]. Whether qualified personnel should interview a study object or a questionnaire be used, remains open for discussion[8,16,22,29]. It may be a point of concern whether a self-assessment questionnaire will make the patient report more complaints than will be revealed by a professional interview[35].

Approximately 10% of patients did not improve or even got worse whereas the condition of 25% improved and the rest was cured. Patients with the most SFG were less likely to be completely cured and this group also had a higher pre-operative symptom score (GSS). Post-operative FGID persisted in 57% of patients and indirect evidence suggests that persistent symptoms were caused mainly by FGID. The main indication for elective cholecystectomy in uncomplicated gallstone disease should be pain attacks. Patients should be informed about the chance of persistent symptoms.

COMMENTS

Background

Patients with gallstones often have various abdominal symptoms that may be caused by the gallstones or are present as a separate condition but with a common physiology. The accompanying abdominal symptoms are called functional gastrointestinal disorders (FGID). Because of its common nature and presence of pain or discomfort it is difficult to separate a functional condition from the gallstone disease itself. Lack of a clear distinction between the two and a poor understanding of the physiology that causes both conditions, especially FGID, makes it difficult to treat these symptoms if they remain after the operation. The article characterizes symptoms caused by gallstone disease in order to define which symptoms remain after removal of the gallbladder. Hope of improving understanding of their character and origin will subsequently have a potential bearing on treatment.

Research frontiers

Current treatment methods may not be satisfactory due to limited insight in physiological mechanisms. Therefore, FGID causes a major health problem with a large amount of sick-leave days. Because of this burden on both patient and society it is important to conduct proper research to gain insight in disease mechanisms and offer effective treatment.

Innovations and breakthroughs

The study tried to characterize gallstone disease according to intensity and frequency of pain attacks as well as concomitant functional symptoms. The pre-

operative condition has then been compared to persisting symptoms after surgery. Such methodical studies of the character of gallstone disease are scarce.

Applications

An understanding of disease expression may give better insight into why complete symptom relief does not occur in some patients after cholecystectomy. Thus, it may be possible in the future to decide which patients will have the greatest chance of cure as well as offer efficient treatment of persisting symptoms after cholecystectomy.

Terminology

Gallstone disease is characterized by bouts of pain or pain attacks in about 85% of patients. The rest have a combination of more consistent pain, strong food intolerance or nausea. FGID is present in about 88% of gallstone patients. This condition may have particular symptoms but a clear-cut physiologic mechanism or organic origin has not been decisively described for it. The diagnosis is sometimes made by exclusion of other diseases. It is difficult to separate clinically from gallstone disease when both are present in the same patient.

Peer review

The authors have nicely analyzed the existing preoperative functional disorder in patients of gallstones to substantiate its correlation with post-operative symptoms.

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BRIEF ARTICLE

Three benefits of microcatheters for retrograde transvenous obliteration of gastric varices

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Abstract

AIM: To evaluate the usefulness of the microcatheter techniques in balloon-occluded retrograde transvenous obliteration (BRTO) of gastric varices.

METHODS: Fifty-six patients with gastric varices underwent BRTOs using microcatheters. A balloon catheter was inserted into gastrorenal or gastrocaval shunts. A microcatheter was navigated close to the varices, and sclerosant was injected into the varices through the microcatheter during balloon occlusion. The next morning, thrombosis of the varices was evaluated by contrast enhanced computed tomography (CE-CT). In patients with incomplete thrombosis of the varices, a second BRTO was performed the following day. Patients were followed up with CE-CT and endoscopy.

RESULTS: In all 56 patients, sclerosant was selectively injected through the microcatheter close to the varices. In 9 patients, microcoil embolization of collateral veins was performed using a microcatheter. In 12 patients with incomplete thrombosis of the varices, additional injection of sclerosant was performed through the microcatheter that remained inserted overnight. Complete thrombosis of the varices was achieved in 51 of 56 patients, and the remaining 5 patients showed incomplete thrombosis of the varices. No recurrence of the varices was found in the successful 51 patients after a median follow up time of 10.5 mo. We experienced one case of liver necrosis, and the other complications were transient.

CONCLUSION: The microcatheter techniques are very effective methods for achieving a higher success rate of BRTO procedures.

Key words: Balloon-occluded retrograde transvenous obliteration; Gastric varices; Microcatheter; Portal hypertension; Ethanolamine oleate

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INTRODUCTION

Balloon-occluded retrograde transvenous obliteration (BRTO) is a treatment for gastric varices that has a high success rate[1-5]. However, there are three major problems with BRTO procedures such as overdose of the sclerosant,

leakage of the sclerosant into the systemic circulation, and incomplete thrombosis of large gastric varices[6-10]. We introduced the microcatheter techniques[11-15] in 1999 to solve these problems, and we have been using three major beneficial techniques for BRTO procedures such as selective injection of the sclerosant, microcoil embolization of collateral veins and additional injection of the sclerosant. Since 1999 we have collected a great deal of data and are now able to accurately report on the long-term results of these techniques in a large number of cases.

MATERIALS AND METHODS

BRTO using 2.9Fr microcatheters was performed in 56 patients with liver cirrhosis-related gastric varices between August 1999 and December 2008. The subjects consisted of 35 males and 21 females, with a mean age of 65.3 years (range: 33-83 years). Liver cirrhosis was associated with hepatitis B in 3 patients, hepatitis C in 29 patients, alcohol in 15 patients, and unknown factors in 9 patients. Acording to the Child-Pugh classification, liver function was evaluated as A in 19 patients, B in 31 patients, and C in 6 patients. Prophylactic BRTO was performed in 31 patients with large tumor-like gastric varices or growing varices in danger of rupture. Elective BRTO was perfomed in 12 patients with a history of hemorrhage related to gastric varices. Emergency BRTO[16,17] was performed in 13 patients within 24 h after hematemesis or tarry stool. Informed consent for BRTO was obtained from all patients.

Gastric varices were confirmed by endoscopy. The presence and diameter of gastrorenal shunt or gastrocaval shunt were evaluated by contrast enhanced computed tomography (CE-CT). An 8Fr sheath(Cobra type; Medikit, Tokyo, Japan) was inserted into the left renal vein or inferior vena cava through the right internal jugular vein or right femoral vein, and a 6Fr balloon catheter(Cobra type; Clinical Supply, Gifu, Japan) was inserted into the gastrorenal shunt or gastrocaval shunt. The balloon diameter was 13 or 20 mm. In patients with a shunt diameter of 13 mm or more, a balloon measuring 20 mm in diameter was used. A 2.9Fr microcatheter was navigated close to the gastric varices. A sclerosant, 5% ethanolamine oleate iopamidol (EOI), was infused slowly and intermittently through a microcatheter during balloon occlusion. 5% EOI was prepared by making a 20 mL solution consisting of 10 mL contrast medium and 10 mL of 10% ethanolamine oleate (Oldamin; Grelan Pharmaceutical, Tokyo, Japan). The infusion of 5% EOI was continued until the entire gastric varices and feeding veins were opacified. The mean volume of sclerosant (5% EOI) was 22.9 mL per one procedure (range: 1.5-47 mL). The balloon occlusion time ranged from 12 to 48 h. To fix the sheath and catheters, sterilized tape (Hogy Medical, Tokyo, Japan) was used. The next morning after the BRTO procedure, thrombosis of gastric varices was evaluated by CE-CT. In patients with incomplete thrombosis after the first BRTO, a second BRTO was performed the following day. After complete thrombosis of gastric varices was confirmed on CE-CT, all catheters were removed. To prevent renal damage due to EOI-induced hemolysis, 4000 units of haptoglobin (Mitsubishi Pharma, Osaka, Japan) was administered intravenously during and after the infusion of EOI in all patients[18,19]. Patients were followed up with endoscopy and CE-CT 1 d, 1 wk and 1, 3, 6 mo after the procedure and every 6 mo thereafter.

RESULTS

In all (100%) of 56 patients, the sclerosant was selectively injected through the microcatheter close to the gastric varices (Figures 1 and 2). In 9 (16%) of 56 patients, microcoil embolization of dilated collateral veins was performed using the microcatheter (Figure 3). In 12 (21%) of 56 patients, CE-CT the next day after the first BRTO showed incomplete thrombosis of the varices, and additional injection of the sclerosant was performed in the second BRTO through the microcatheter which remained inserted overnight (Figure 4). Complete thrombosis of the varices was achieved in 51 of 56 patients after all BRTO procedures, and the remaining 5 patients showed incomplete thrombosis of the varices. Endoscopic treatments were performed in 4 of the 5 patients[20-24], and a surgical treatment was performed in the other patient. No cases of recurrence or variceal bleeding of the gastric varices were found in the successful 51 patients after a median follow up time of 10.5 mo (range one day-7 years). Esophageal varices with red color sign appeared in 5 of the 51 patients[25-28]. Red color sign indicates a high risk of variceal bleeding[29]. These patients' varices were treated by endoscopic treatment.

Most complications were transient and minor. These include: hematuria due to the sclerosant (8 of 56 patients), high fever (8 of 56), abdominal pain (5 of 56), elevation of blood pressure during infusion of the sclerosant (3 of 56), pleural effusion (35 of 56), ascites (33 of 56)[30], and extravasation of the sclerosant during the procedure (3 of 56). In the three patients with extravasation, BRTO was continued, and complete thrombosis of the varices was achieved in 2 patients. We experienced one case of liver necrosis after the BRTO procedure[31]. No other major complications such as renal failure, pulmonary embolism, or acute respiratory distress syndrome (ARDS) were experienced.

DISCUSSION

Microcatheters have three major benefits in BRTO for gastric varices. The first benefit is a selective injection of the sclerosant through a microcatheter[11-13]. Infusion of the sclerosant with a microcatheter, which is inserted close to the gastric varices, enables a decrease in the dose of the sclerosant, preventing sclerosant-related complications. We consider that the optimal volume of the sclerosant used for one BRTO procedure is 40 mL or less. To decrease the sclerosant volume of 5% EOI, 50% glucose solution may be infused before injection of 5% EOI during BRTO[32].

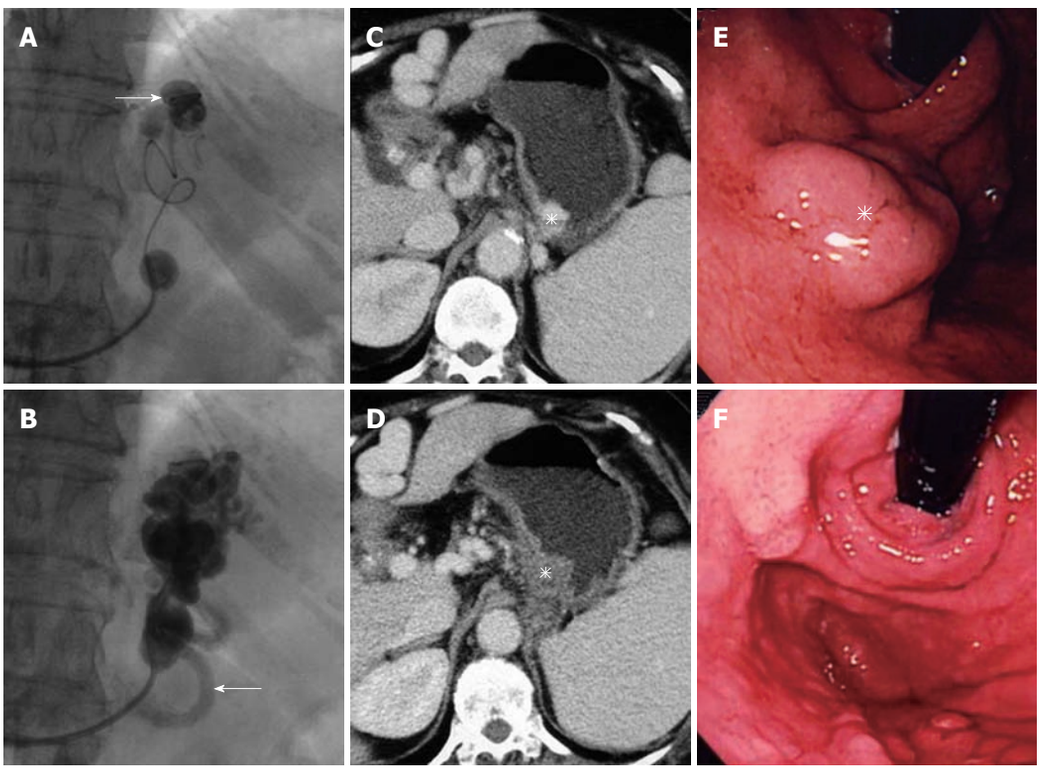

Figure 1 Selective injection of the sclerosant. A: A microcatheter is navigated close to the gastric varices, and the sclerosant is selectively injected through the microcatheter (arrow); B: The gastric varices and gastrorenal shunt are fully filled with the sclerosant with contrast medium, and the afferent vein (arrow) is opacified; C: Contrast-enhanced computed tomography (CE-CT) before balloon-occluded retrograde transvenous obliteration (BRTO) shows gastric varices (asterisk); D: CE-CT one week after BRTO shows complete thrombosis of the varices (asterisk); E: Endoscopy before BRTO shows tumor-like varices (asterisk) in the fornix of the stomach; F: Endoscopy 3 mo after BRTO shows complete disappearance of the varices.

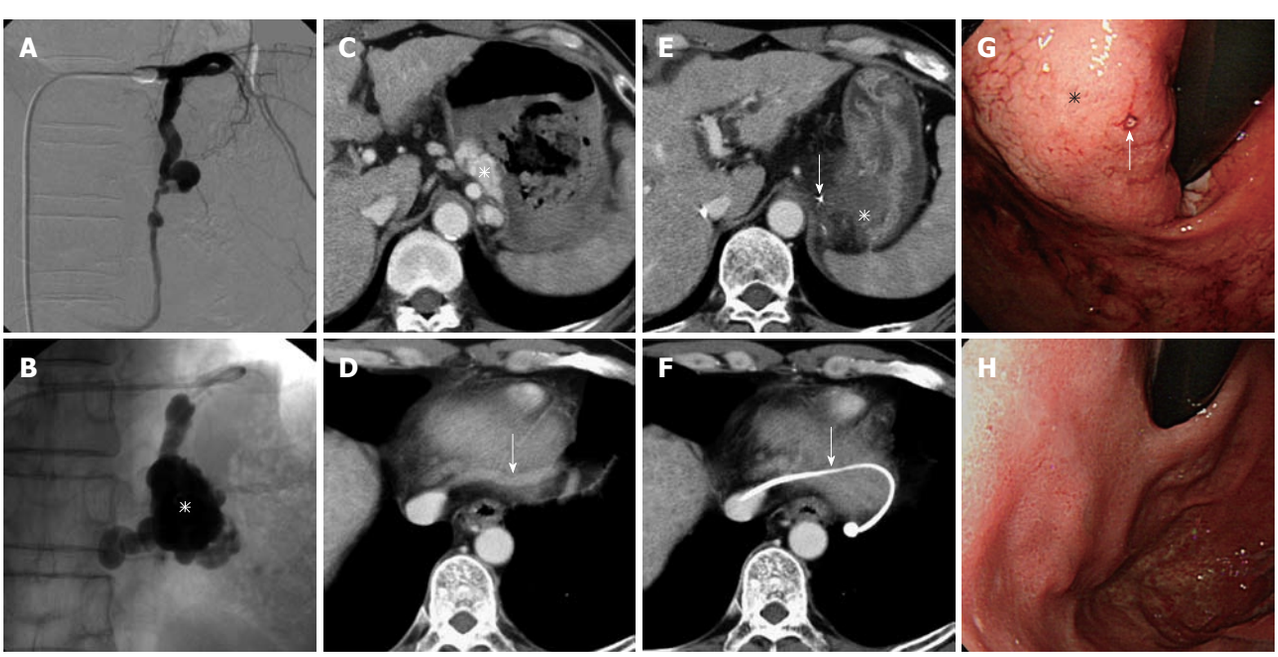

Figure 2 Selective injection of the sclerosant. A: A balloon catheter is inserted into the gastrocaval shunt. Balloon-occluded venography shows no gastric varices; B: The balloon catheter is advanced further into the shunt, and the sclerosant is selectively injected through the microcatheter which is navigated close to the gastric varices. The varices (asterisk) are opacified sufficiently; C: Contrast-enhanced computed tomography (CE-CT) shows the varices (asterisk) and a large amount of hematomas in the stomach; D: The gastrocaval shunt (arrow) flows into the inferior vena cava; E: CE-CT next day shows complete thrombosis of the gastric varices (asterisk) and the tip of the microcatheter (arrow) close to the varices; F: CE-CT shows the balloon catheter in the shunt (arrow); G: Endoscopy before balloon-occluded retrograde transvenous obliteration (BRTO) shows large gastric varices (asterisk) with a bleeding site (arrow); H: Endoscopy 3 mo after BRTO shows complete disappearance of the varices.

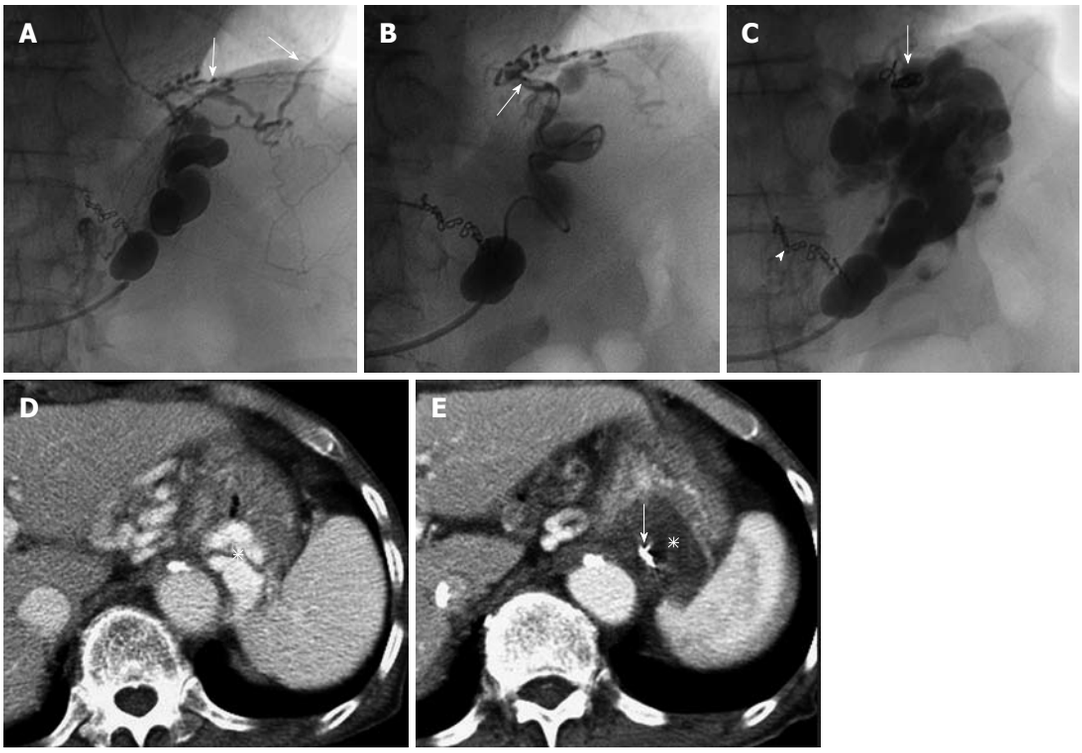

Figure 3 Microcoil embolization of collateral veins. A: Pericardiophrenic veins (arrows) develop as collateral draining veins; B: A microcatheter (arrow) is navigated into the pericardiophrenic vein and microcoil embolization is performed; C: The sclerosant is selectively injected through the microcatheter which is withdrawn a little, and the gastric varices are opacified sufficiently. Microcoils (arrow) from embolization and surgical clips from previous operation of gastric cancer. (arrowhead) are seen; D: Contrast-enhanced computed tomography (CE-CT) before balloon-occluded retrograde transvenous obliteration (BRTO) shows gastric varices (asterisk); E: CE-CT next day after BRTO shows complete thrombosis of the varices (asterisk) and microcoils close to the varices (arrow).

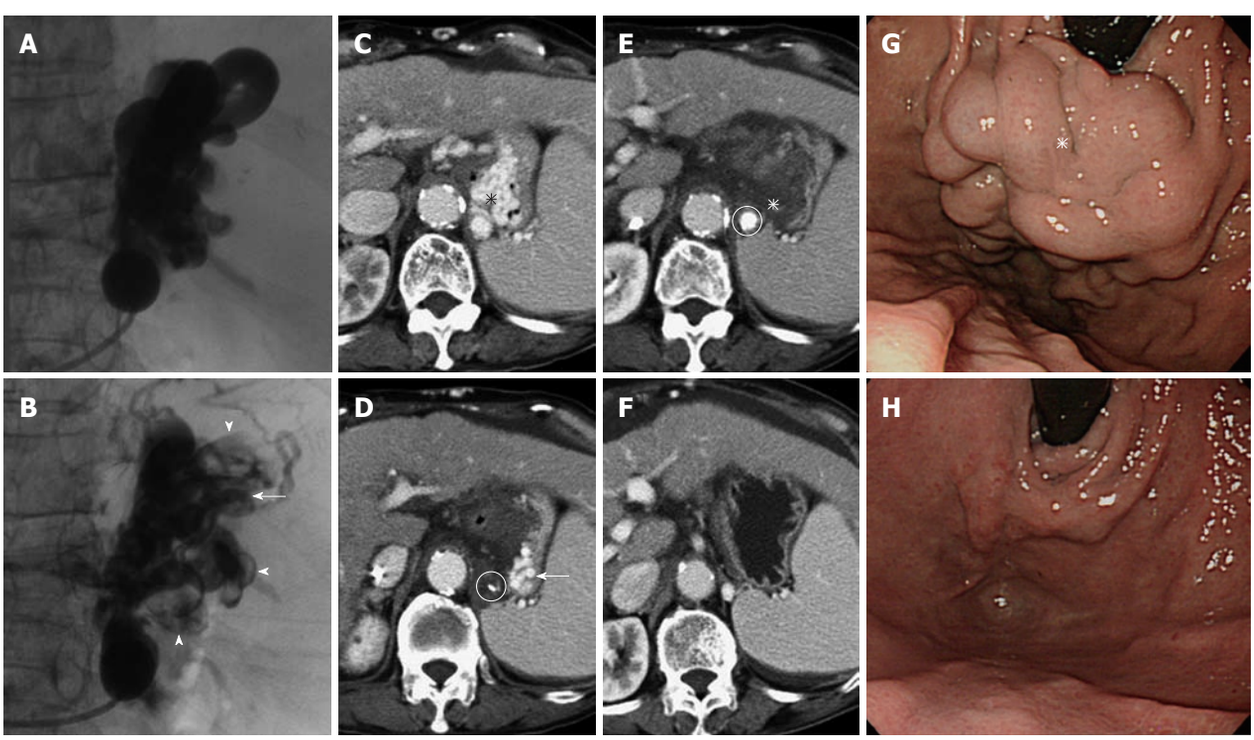

Figure 4 Additional injection of the sclerosant. A: Fluoroscopic image obtained during the first balloon-occluded retrograde transvenous obliteration (BRTO) shows full opacification of the gastric varices and gastrorenal shunt; B: Fluoroscopic image obtained during the second BRTO (next day) shows partial opacification of the varices and shunt, suggesting residual varices (arrow) and thrombosis of the varices and shunt (arrowheads); C: Contrast-enhanced computed tomography (CE-CT) before BRTO shows large varices (asterisk); D: CE-CT after the first BRTO shows residual varices (arrow) in the lateral portion of the stomach. The microcatheter tip (circle) is in the gastrorenal shunt close to the varices; E: CE-CT after the second BRTO shows complete thrombosis of the varices (asterisk). The sclerosant with contrast medium (circle) is detected in the gastrorenal shunt; F: CE-CT 3 mo after BRTO shows complete disappearance of the varices; G: Endoscopy before BRTO shows bulky varices (asterisk); H: Endoscopy 3 mo after BRTO shows complete disappearance of the varices.

The second benefit is a microcoil embolization of dilated collateral veins[33] using a microcatheter[11,12,14]. Obliteration of collateral veins prevents renal failure, pulmonary embolism, and ARDS induced by leakage of the sclerosant into the systemic circulation. Haptoglobin was intravenously administered as a counteragent of ethanolamine oleate, which is a sclerosant that damages the endothelial cell of the vessel and induces thrombus formation in the vessel.

The third benefit is an additional injection of the sclerosant through the microcatheter that remained inserted overnight[15]. To achieve complete thrombosis of gastric varices, the balloon occlusion time was prolonged from 30 min (original BRTO) to 12 h or more[1]. After a complete thrombosis of gastric varices was confirmed on CE-CT done the next morning after the first BRTO, all catheters were removed. When complete thrombosis of gastric varices was not achieved, a second BRTO was performed, and additional sclerosant was injected through the microcatheter. Insertion of a microcatheter close to the gastric varices until the next day allows for an additional injection of the sclerosant into the varices through the microcatheter, even when occlusion of a shunt occurs.

Another minor benefit is that microcatheters can be a safer and more accurate guidance tool for balloon catheters than the 0.035 inch guidewires. The stiff guidewires sometimes induce venous damage. On the other hand, it' s easy to insert a soft microcatheter into the shunts and advance a balloon catheter into the shunts over the micorocatheter and microguidewire, because we can check the position of the microcatheter tip by test injection of the contrast material.

In the Kanagawa *et al*[1] on use of BRTO without the microcatheter technique, complete eradication of gastric varices was not achieved after a single BRTO procedure in 7 (22%) of 32 patients. This is compatible with our results that show 21% of patients with incomplete thrombosis of the varices and 16% of patients having microcoil embolization.

BRTO procedures for gastric varices may be difficult to conduct when varices lack a gastrorenal shunt[34-37]. However, gastric varices without the gastrorenal shunt are rare.

We experienced one case of liver necrosis. It is supposed that the liver necrosis was due to leakage of the sclerosant into the portal vein through afferent veins. So we must be careful in order to prevent leakage of the sclerosant into the portal vein.

Esophageal varices with red color sign appeared in 5 patiets[25-28]. Occlusion of a gastrorenal shunt and/or gastrocaval shunt may have induced esophageal varices as another collateral route. Esophageal varices can be readily treated by endoscopic treatment. Therefore, the status of esophageal varices should be endoscopically checked at 6-month intervals after BRTO.

Three major beneficial techniques of microcatheters for BRTO of gastric varices are selective injection of the sclerosant, microcoil embolization of collateral veins and additional injection of the sclerosant. Microcatheters are useful for achieving a higher success rate of BRTO procedures.

COMMENTS

Background

Gastric varices have a larger blood flow compared with esophageal varices, so when they are ruptured, there is a high mortality rate. Therefore, prophylactic treatment is necessary in patients with gastric varices in danger of rupture. Balloon-occluded retrograde transvenous obliteration (BRTO), **is a treatment for** gastric varices that is minimally invasive and has a high success rate. However, there are three major problems with BRTO procedures such as overdose of the sclerosant, leakage of the sclerosant into the systemic circulation, and incomplete thrombosis of large gastric varices. We introduced the microcatheter techniques to solve these problems

Innovations and breakthroughs

Microcatheters have three major benefits in BRTO for gastric varices. The first benefit is a selective injection of the sclerosant through a microcatheter. The second benefit is a microcoil embolization of dilated collateral veins using a microcatheter. The third benefit is additional injection of the sclerosant through the microcatheter that remained inserted overnight. When complete thrombosis of gastric varices was not achieved, a second BRTO was performed, and additional sclerosant was injected through the microcatheter.

Applications

Patients with large gastric varices and/or dilated collateral veins can be treated with BRTO procedures using the microcatheter techniques.

Peer review

In this study, the authors described three major beneficial techniques of microcatheters for BRTO of gastric varices. Microcatheters are useful for achieving a higher success rate of BRTO procedures.

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BRIEF ARTICLE

Branched-chain amino acid treatment before transcatheter arterial chemoembolization for hepatocellular carcinoma

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Abstract

AIM: To examine the significance of branched-chain amino acid (BCAA) treatment before transcatheter arterial chemoembolization (TACE) for hepatocellular carcinoma (HCC).

METHODS: This study included 99 patients who underwent TACE therapy for HCC at our hospital and were followed up without treatment for at least 6 mo between January 2004 and January 2010. They were divided into 2 groups: those receiving BCAA granules (n = 40) or regular diet (n = 59, control). Data obtained were retrospectively analyzed (prior to TACE, and 1 wk, 1, 3, and 6 mo after TACE) in terms of nutritional condition and clinical laboratory parameters (serum albumin level and Child-Pugh score), both of which are determinants of hepatic functional reserve.

RESULTS: The BCAA group comprised 27 males and 13 females with a mean age of 69.9 ± 8.8 years. The patients of the BCAA group were classified as follows: Child-Pugh A/B/C in 22/15/3 patients, and Stage Ⅱ/Ⅲ/ⅣA HCC in 12/23/5 patients, respectively. The control group comprised 32 males and 27 females with a mean age of 73.2 ± 10.1 years. In the control group, 9 patients had chronic hepatitis, Child-Pugh A/B/C in 39/10/1 patients, and Stage Ⅰ/Ⅱ/Ⅲ/ⅣA HCC in 1/11/35/12 patients, respectively. Overall, both serum albumin level and Child-Pugh score improved significantly in the BCAA group as compared with the control 3 and 6 mo after TACE (P < 0.05). Further analysis was performed by the following categorization: (1) child-Pugh classification; (2) liver cirrhosis subgroup with a serum albumin level > 3.5 g/dL; and (3) epirubicin dose. A similar trend indicating a significant improvement of all variables in the BCAA group was noted (P < 0.05).

CONCLUSION: Treatment with BCAA granules in patients who have undergone TACE for HCC is considered useful to maintain their hepatic functional reserve.

Key words: Hepatocellular carcinoma; Branched-chain amino acid granules; Transcatheter arterial chemoembolization; Liver function; Improvement; Cirrhosis; Protein-energy malnutrition

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Nishikawa H, Osaki Y, Inuzuka T, Takeda H, Nakajima J, Matsuda F, Henmi S, Sakamoto A, Ishikawa T, Saito S, Kita R, Kimura T. Branched-chain amino acid treatment before transcatheter arterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2012; 18(12): 1379-1384 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i12/1379.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i12.1379

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common

carcinoma worldwide[1]. Treatment for HCC varies depending on the disease stage and liver function, and includes radiofrequency ablation, percutaneous ethanol injection therapy, hepatic resection, liver transplantation, transcatheter arterial chemoembolization (TACE), and molecular target therapy[2-4].

TACE is a procedure whereby an embolizing agent is injected into the hepatic artery to deprive the tumor of its major nutrient source *via* embolization of the nutrient artery, resulting in ischemic necrosis of the tumor. Hepatic arterial embolization, which had been used until early in the 1990s, is divided into two treatment methods: injection of an embolizing agent after intra-arterial injection of an anticancer drug and intra-arterial injection of a mixture of an embolizing agent and an anticancer drug[5,6]. Subsequently, it was revealed that an oil contrast medium or iodized oil (Lipiodol) accumulates within the tumor after injection. This led to introduction of TACE, in which an embolizing agent is injected after injection of a mixture of Lipiodol and an anticancer drug (Lipiodol emulsion)[7,8]. Until the middle of the 1990s TACE had been performed in a large majority of patients with unresectable HCC. With the subsequent introduction of local treatment, however, TACE is now mainly indicated for treatment of an HCC measuring 3 to 5 cm in diameter or treatment of 4 or more HCCs less than 3 cm in diameter that are both unresectable and not indicated for local treatment.

Takayasu *et al*[9] reported that independent prognostic factors in relation to survival in patients who underwent TACE include (1) degree of hepatic damage; (2) tumor staging; and (3) serum α-fetoprotein level, and recommended TACE, which can sufficiently maintain the volume ratio of a chemoembolized tumorous liver to the entire tumor-free liver as well as of residual hepatic functional reserve, while emphasizing the importance of maintenance of hepatic functional reserve in these patients.

Branched-chain amino acids (BCAAs) are three amino acids possessing branched side chains (i.e., valine, leucine, and isoleucine). Patients with liver cirrhosis are known to have decreased plasma BCAA levels, which can lead to protein-energy malnutrition (PEM). PEM is associated with a high morbidity and mortality due to an increased risk of life-threatening complications, resulting in poor survival and quality of life (QoL)[10].

A considerable proportion of patients with HCC have concurrent liver cirrhosis. In those patients with underlying PEM, interventional therapy such as TACE may further worsen their nutritional condition and even occasionally cause development of ascites and jaundice, resulting in an irreversible outcome[11].

Supplementation with BCAAs in patients with liver disorder has been attracting attention. BCAA treatment can correct malnutrition associated with liver cirrhosis in animals and humans[12-14], and long-term nutritional BCAA supplementation may also be useful for prevention of hepatic failure while it also improves surrogate markers in patients with advanced cirrhosis[15,16]. BCAA supplementation is also effective in down-regulating protein metabolism in liver cirrhosis patients by reducing ammonia (NH_3) level, thus improving the nitrogen balance and resulting in better clinical outcomes[17,18]. The mechanism underlying these beneficial effects of BCAAs might be mediated by stimulation of hepatocyte growth factor activity that induces liver regeneration[19]. Therefore, nutritional support may play an important role in management of liver cirrhosis in patients with unresectable HCC. Studies dealing with the effect of treatment with BCAA granules before TACE in patients with HCC, nevertheless, are few as yet to our knowledge. This study was thus performed to investigate the significance of BCAA treatment in HCC patients who had undergone TACE.

MATERIALS AND METHODS

Patients

This retrospective study included 99 patients who underwent TACE alone for treatment of HCC at our hospital and were followed up thereafter without treatment for at least 6 mo between January 2004 and January 2010. Patients were divided into two groups: those receiving BCAA treatment (n = 40) or regular diet (n = 59, control). BCAA therapy had been started at least one month before the day TACE was performed, and treatment compliance was good in all patients receiving BCAAs.

Diagnosis of hepatocellular carcinoma

Dynamic computed tomography (CT) and abdominal echography were performed in all patients. A lesion visualized as a tumor blush in the early phase scan and as a defect area in the late phase scan on dynamic CT was diagnosed as HCC. It has been verified that such lesions appear as blushes on CT hepatic angiography and as defect areas on CT arterial portography during TACE. Two radiologists proficient in diagnostic imaging of the liver made a diagnosis of HCC. No pathological examination was conducted.

Branched-chain amino acid granules

BCAA granules, containing 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine per sachet, were orally administered to subjects at a dose of one sachet three times daily after meals. The control patients received no such treatment.

Transcatheter arterial chemoembolization procedure

Written informed consent was obtained from each patient prior to TACE. The protocol for TACE was approved by the independent ethics committee of the hospital. TACE for HCC was performed in conformity with Japanese guidelines for this therapy[20] and consisted of catheterization *via* the femoral artery with super-selective cannulation to the hepatic artery feeding the target HCC. Farmorubicin (epirubicin hydrochloride, Pfizer) emulsion was infused at 10 to 60 mg, and Lipiodol (iodine addition products of ethyl esters of fatty acids obtained from pop-

Table 1 Baseline characteristics of study groups (mean ± SD)

	BCAA group (n = 40)	Control group (n = 59)	P value
Gender			
Male	27	32	0.215
Female	13	27	
Age (yr)	69.9 ± 8.8	73.2 ± 10.1	0.092
Etiology of liver disease			
Chronic hepatitis C	28	43	0.287
Chronic hepatitis B	2	8	
Non B non C	10	10	
Child-Pugh classification			
Chronic hepatitis	0	9	0.006
Child-Pugh A	22	39	
Child-Pugh B	15	10	
Child-Pugh C	3	1	
WBC ($\times 10^3/\mu L$)	38.2 ± 10.8	44.7 ± 16.0	0.082
Hb (g/dL)	11.9 ± 1.8	12.5 ± 1.7	0.091
Platelet ($\times 10^4/mm^3$)	10.2 ± 9.4	11.4 ± 4.9	0.431
Alb (g/dL)	3.32 ± 0.50	3.74 ± 0.51	< 0.001
T-Bil (mg/dL)	1.28 ± 0.81	1.05 ± 0.63	0.123
PT (%)	77.5 ± 14.1	85.9 ± 17.3	0.012
AST (IU/L)	65.8 ± 39.6	73.8 ± 56.4	0.445
ALT (IU/L)	48.0 ± 38.8	54.2 ± 39.0	0.438
AFP (ng/mL)	626.1 ± 2009.8	1109.2 ± 2652.5	0.331
PIVKAII (mAU/mL)	1471.7 ± 5033.5	3421.5 ± 8211.2	0.183
HCC Stage			
Stage Ⅰ	0	1	0.412
Stage Ⅱ	12	11	
Stage Ⅲ	23	35	
Stage Ⅳa	5	12	
Max tumor size (cm)	3.34 ± 1.67	3.59 ± 1.47	0.422
Epirubicin dose (mg)	34.8 ± 10.4	39.5 ± 9.2	0.024

WBC: White blood cell; Hb: Hemoglobin; Alb: Albumin; T-Bil: Total bilirubin; PT: Prothrombin time; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; PIVKAⅡ: Protein induced vitamin K absence or antagonist Ⅱ; HCC: Hepatocellular carcinoma; BCAA: Branched-chain amino acids.

py seed oil; Mitsui, Japan) was also injected at 2 to 10 mL according to the tumor size and tumor number. This was followed by embolization with gelatin (Spongel; Yamanouchi, Japan), which was injected slowly to prevent reflux into untreated segments. The sites of injection of the embolizing agents were segmental or subsegmental in all patients.

Follow-up after transcatheter arterial chemoembolization

At 1 wk and 1, 3 and 6 mo after TACE, patients underwent hematological and blood biochemical tests and were assessed for their hepatic functional reserve and development of any adverse events. Dynamic CT was carried out to assess for any ascites or recurrence of HCC at 1, 3 and 6 mo after TACE.

Statistical analysis

Student t test, χ^2 test and Fisher's exact test were used to compare data between BCAA patients and the control. Serum albumin level and Child-Pugh score constituted parameters for assessment of hepatic functional reserve. Absolute changes in serum albumin level observed at 1 wk and 1, 3 and 6 mo after TACE were compared between the two groups and evaluated using Student t test, and the absolute change was defined as the difference found at each assessment time point from the baseline (pre-TACE level). Changes in Child-Pugh score were also evaluated similarly using Student t test at 1, 3 and 6 mo after TACE.

Data were analyzed using SPSS software, version 9.0 (SPSS Inc., Chicago, IL, United States) for Microsoft Windows. Data are expressed as mean ± SD. Values of $P < 0.05$ were considered to be statistically significant.

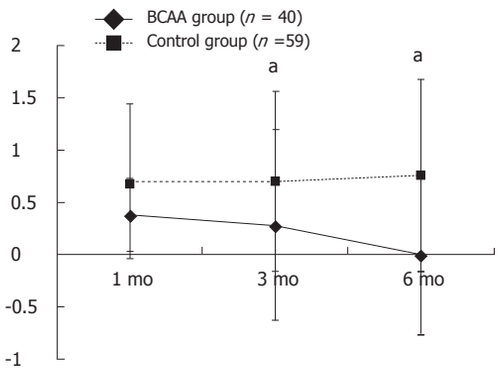

Figure 1 Overall comparison of changes in Child-Pugh score between the branched-chain amino acids group and the control group over time. There was a significant difference in changes in Child-Pugh score 3 and 6 mo after transcatheter arterial chemoembolization. $^aP < 0.05$ *vs* control group. BCAA: Branched-chain amino acid.

RESULTS

Patient demographic characteristics are summarized in Table 1. Significant differences were noted for the following parameters: Child-Pugh score, serum albumin level, prothrombin time, and dose of epirubicin at the time of TACE. A patient in the control group had stage Ⅰ HCC, for which percutaneous therapy is indicated, but TACE alone was performed because the patient refused percutaneous therapy.

Overall comparison of hepatic functional reserve between the branched-chain amino acid group and the control group over time

A significant difference in serum albumin level was observed at all assessment time points ($P < 0.05$). Also, there was a significant difference in Child-Pugh score 3 and 6 mo after TACE ($P < 0.05$) (Table 1, and Figure 1).

The categorized analysis results are presented below.

Comparison of hepatic functional reserve in Child A patients

There were 22 Child A patients in the BCAA group and 39 in the control group. A significant difference was noted in serum albumin level 1, 3 and 6 mo after TACE and in Child-Pugh score 3 and 6 mo after TACE ($P < 0.05$) (Table 1, and Figure 2A).

Comparison of hepatic functional reserve in Child B patients

There were 15 Child B patients in the BCAA group and 10 in the control group. A significant difference was

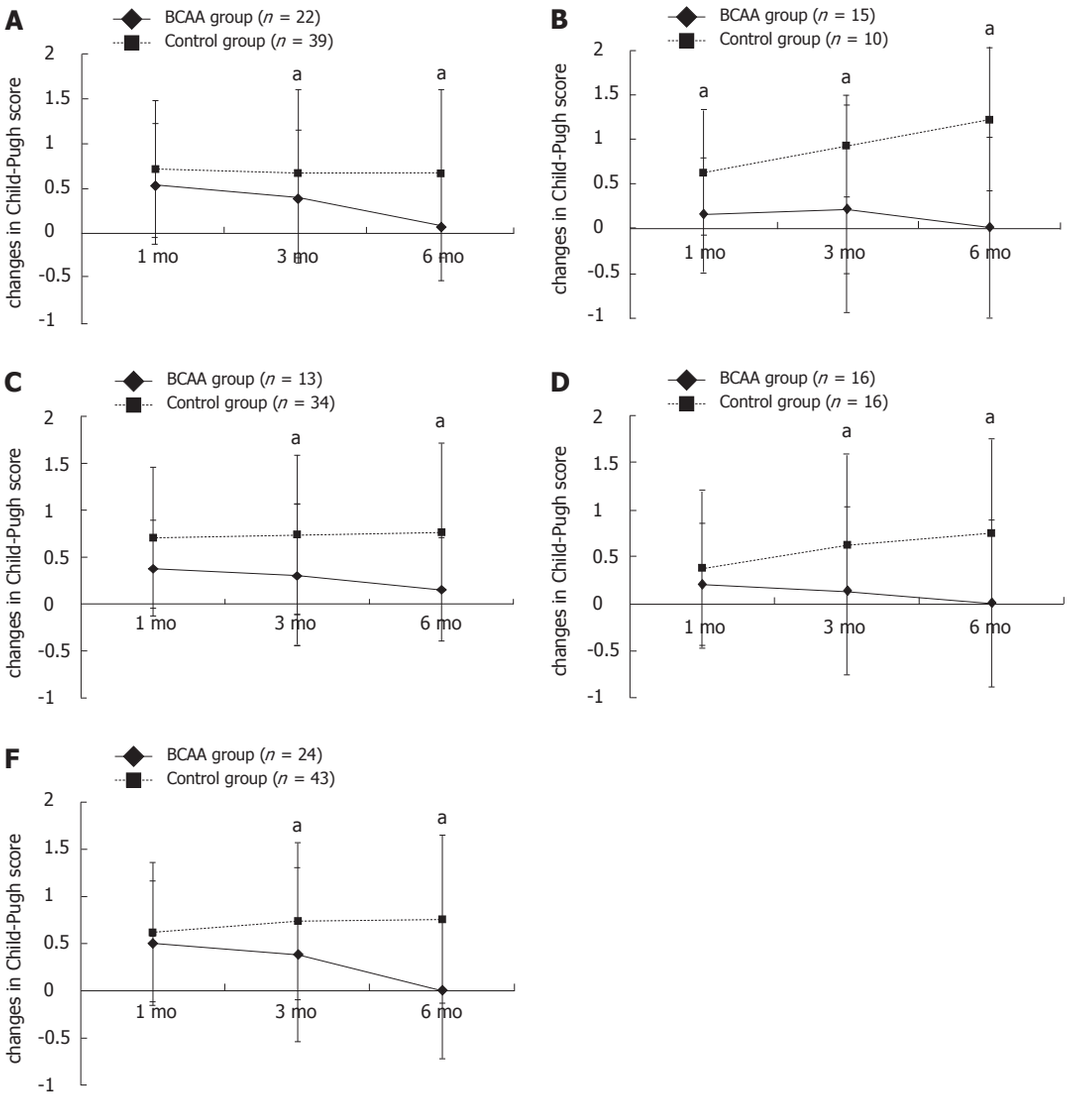

Figure 2 Comparison of changes in Child-Pugh score in Child A and Child B patients: patients with a serum albumin level of 3.5 g/dL or more, low-dose epirubicin subgroups, high-dose epirubicin subgroups. A: A significant difference was noted in changes in Child-Pugh score 3 and 6 mo after transcatheter arterial chemoembolization; B: A significant difference was noted in changes in Child-Pugh score 1, 3 and 6 mo after transcatheter arterial chemoembolization; C: A significant difference was observed in changes in Child-Pugh score 3 and 6 mo after TACE; D: A significant difference was noted in changes in Child-Pugh score 3 and 6 mo after TACE; E: A significant difference was noted in changes in Child-Pugh score 3 and 6 mo after TACE. BCAA: Branched-chain amino acids; TACE: Transcatheter arterial chemoembolization. $^{a}P < 0.05$ *vs* control group.

noted in serum albumin level 3 and 6 mo after TACE and in Child-Pugh score 1, 3 and 6 mo after TACE ($P < 0.05$) (Table 1 and Figure 2B).

Comparison in patients with a serum albumin level of 3.5 g/dL or more

There were 13 and 34 patients who fell in this category in the BCAA group and the control group, respectively. A significant difference was observed in both serum albumin level and Child-Pugh score 3 and 6 mo after TACE ($P < 0.05$) (Table 1, and Figure 2C).

As it is thought that antineoplastic agents used during TACE may cause hepatic impairment in a dose-dependent fashion, the data were further evaluated in patients classified into two subgroups: those treated with low-dose epirubicin (less than 40 mg) or a high-dose epirubicin (40 mg or more).

Comparison in low-dose epirubicin subgroups

Sixteen patients each received low-dose epirubicin in the BCAA group and the control group. Serum albumin level was significantly different 1, 3 and 6 mo after TACE and Child-Pugh score 3 and 6 mo after TACE ($P < 0.05$) (Table 1 and Figure 2D).

Comparison in high-dose epirubicin subgroups

Twenty-four and 43 patients received high-dose epirubicin in the BCAA group and the control group, respectively. A significant difference was noted in serum albumin level at all assessment time points and in Child-Pugh score 3 and 6 mo after TACE ($P < 0.05$) (Table 1 and Figure 2E).

DISCUSSION

PEM occurs frequently in patients with liver cirrhosis and represents an important predictive factor for the prognosis of liver cirrhosis patients with HCC [18,21]. Supplementation with BCAA formula is reportedly useful for improving PEM and QoL in these patients. However, few studies have assessed the importance of such nutritional intervention in patients with HCC who underwent nonsurgical therapies such as TACE. The purpose of the present study was to investigate to what extent BCAA treatment can contribute to maintaining hepatic functional reserve in HCC patients after TACE.

A significant difference was observed in the overall patient population in terms of change in serum albumin level at all assessment time points. As seen in Table 1, hepatic functional reserve was relatively well maintained in the control group; therefore, anticancer chemotherapy was given at relatively high doses (60% of patients treated with BCAA received epirubicin at 40 mg or more whereas the corresponding percentage for the control group was 72.9%). Patients receiving high-dose anticancer chemotherapy are often unable to sufficiently ingest food over several weeks after TACE. This may account for lower serum albumin levels observed in the control group compared with the BCAA group. Other possible causes of decreased serum albumin levels after TACE include (1) impaired ability of the liver to synthesize serum albumin due to decreased hepatocyte count; (2) inhibition of the synthesis of albumin by inflammatory cytokines; and (3) leakage of albumin due to inflammation of the cauterized areas[22,23].

The assessments in Child-Pugh A patients revealed a significant difference in serum albumin level 1, 3 and 6 mo after TACE and in Child-Pugh score 3 and 6 mo after TACE. TACE is best indicated for Child-Pugh A HCC. In patients undergoing TACE, caution should be exercised to minimize depression of hepatic functional reserve in preparation for the next treatment session. The above results thus suggest the usefulness of BCAA treatment in this regard.

The assessments in the Child-Pugh B subgroup showed a significant difference in Child-Pugh score 1, 3 and 6 mo after TACE. Once hepatic functional reserve has worsened from Child-Pugh B to Child-Pugh C following TACE, the next TACE cannot be performed according to the Barcelona Clinic Liver Cancer guidelines[24]. Therefore, particular caution should be exercised in maintaining hepatic functional reserve at the time of TACE in patients with Child-Pugh B HCC, indicating the indispensability of BCAA therapy.

In Japan, BCAA granules are indicated for the treatment of liver cirrhosis in patients with a serum albumin level of 3.5 g/dL or less. However, conversely, the present study demonstrated similar results between patients with a serum albumin level of more than 3.5 g/dL and those in other categories of serum albumin level. Therefore, treatment with BCAA proved to improve hepatic functional reserve even in cirrhotic patients with HCC whose serum albumin level exceeds 3.5 g/dL. It is thus recommended to actively provide BCAA treatment in such patients.

There was a conspicuous difference between the BCAA and control groups in respect of response to BCAA therapy when assessed in patients receiving high-dose epirubicin compared to those treated with low-dose epirubicin. TACE may cause a marked damage to the liver in HCC patients, eventually leading to a considerable impact on their hepatic functional reserve[9]. BCAA treatment is thus recommended at sufficient doses prior to TACE in patients with advanced HCC in whom high-dose anticancer chemotherapy is anticipated.

TACE is often repeated because a single session of therapy seldom provides complete necrosis of a tumor. The procedure is commonly repeated once every 2 to 3 mo[25-27]. In the present study, however, many patients failed to attain recovery of hepatic functional reserve to a pre-TACE level, particularly in the control group, within 2 to 3 mo of TACE. It is thus estimated that every repeated session of TACE may worsen hepatic functional reserve and thereby shorten the prognosis for survival. Treatment with BCAA would therefore be essential in order to allow for providing TACE periodically while securely maintaining hepatic functional reserve.

One of the findings commonly noted in regard to all the variables assessed in this study is that a significantly greater improvement was noted in both serum albumin level and Child-Pugh score for the BCAA group 6 mo after TACE in comparison to the control group. What is suggested by this fact is simply the usefulness of long-term BCAA treatment prior to TACE. It is also important that patients should be fully instructed on the use of BCAA granules to maintain their treatment compliance.

The present study has several limitations. Firstly, it is a retrospective study. Furthermore, there was a bias in patient demographic characteristics between the BCAA and control groups since BCAA is usually used for patients showing low serum albumin levels. Therefore, pertinent data were evaluated for improvement or exacerbation using absolute serum albumin change as a parameter. The present study did not include assessment for the prognosis for survival, which should be addressed by a prospective study using comparable demographic characteristics among patients.

In conclusion, treatment with BCAAs before TACE in HCC patients is extremely useful in maintaining their hepatic functional reserve.

COMMENTS

Background

Patients with hepatocellular carcinoma (HCC) due to liver cirrhosis are known to have decreased plasma branched-chain amino acid (BCAA) levels, which can lead to protein-energy malnutrition (PEM). BCAA treatment can correct malnutrition associated with liver cirrhosis.

Research frontiers

Studies dealing with the effect of treatment with BCAA granules before transcatheter arterial chemoembolization (TACE) in patients with HCC are few as

yet. In this study, the authors analyzed the effect of BCAA treatment before TACE for HCC patients.

Innovations and breakthroughs

Recent studies imply that by BCAA supplementation, malnutrition associated with liver cirrhosis is corrected and liver function improves. The present study shows that in HCC patients who underwent TACE, liver function was maintained by BCAA supplementation.

Applications

This study emphasizes the importance of BCAA treatment before TACE for HCC patients with regard to maintaining liver function.

Peer review

This is a very good and novel study in which authors analyze the effect of BCAA treatment before TACE for HCC patients. The results are interesting and suggest the usefulness of BCAA treatment before TACE in HCC patients in maintaining their hepatic functional reserve.

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BRIEF ARTICLE

Assessment of disease progression in patients with transfusion-associated chronic hepatitis C using transient elastography

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Abstract

AIM: To evaluate the relationship between liver stiffness and duration of infection in blood transfusion-associated hepatitis C virus (HCV) patients with or without hepatocellular carcinoma (HCC).

METHODS: Between December 2006 and June 2008, a total of 524 transfusion-associated HCV-RNA positive patients with or without HCC were enrolled. Liver stiffness was obtained noninvasively by using Fibroscan (Echosens, Paris, France). The date of blood transfusion was obtained by interview. Duration of infection was derived from the interval between the date of blood transfusion and the date of liver stiffness measurement (LSM). Patients were stratified into four groups based on the duration of infection (17-29 years; 30-39 years; 40-49 years; and 50-70 years). The difference in liver stiffness between patients with and without HCC was assessed in each group. Multiple linear regression analysis was used to determine the factors associated with liver stiffness.

RESULTS: A total of 524 patients underwent LSM. Eight patients were excluded because of unsuccessful measurements. Thus 516 patients were included in the current analysis (225 with HCC and 291 without). The patients were 244 men and 272 women, with a mean age of 67.8 ± 9.5 years. The median liver stiffness was 14.3 kPa (25.8 in HCC group and 7.6 in non-HCC group). The patients who developed HCC in short duration of infection were male dominant, having lower platelet count, with a history of heavier alcohol consumption, showing higher liver stiffness, and receiving blood transfusion at an old age. Liver stiffness was positively correlated with duration of infection in patients without HCC (r = 0.132, P = 0.024) but not in patients with HCC (r = -0.103, P = 0.123). Liver stiffness was significantly higher in patients with HCC than in those without in each duration group (P < 0.0001). The factors significantly associated with high liver stiffness in multiple regression were age at blood transfusion (P < 0.0001), duration of infection (P = 0.0015), and heavy alcohol consumption (P = 0.043)

CONCLUSION: Although liver stiffness gradually increases over time, HCC develops in patients with high stiffness value regardless of the duration of infection.

Key words: Transfusion-associated hepatitis C; Transient elastography; Hepatocellular carcinoma; Liver stiffness; Ultrasonography; Liver fibrosis

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Masuzaki R, Tateishi R, Yoshida H, Arano T, Uchino K, Enooku K, Goto E, Nakagawa H, Asaoka Y, Kondo Y, Goto T, Ikeda H, Shiina S, Omata M, Koike K. Assessment of disease progression in patients with transfusion-associated chronic hepatitis C using transient elastography. *World J Gastroenterol* 2012; 18(12): 1385-1390 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i12/1385.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i12.1385

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, presenting serious public health problems worldwide[1,2]. HCV infection generally shows an asymptomatic onset and slow fibrosis progression, with cirrhosis developing after 20-50 years[3-7]. Progression of disease is known to depend on patients' characteristics at the onset of infection[8-12]. Infection at old age, male gender and heavy alcohol consumption are reported to be independently associated with rapid disease progression.

The onset of HCV infection can be reliably estimated in transfusion-associated chronic hepatitis C patients, in contrast to repeating injecting-drug users. In Japan, about 40% of chronic hepatitis C patients and HCV-related HCC patients have a history of blood transfusion typically in 1950s and 1960s[13], when blood supply depended on paid blood donors. Not a few of the blood donors were also injecting-drug users, mainly methamphetamine, among whom HCV spread first after the end of World War Ⅱ. Viral spread started to decline in Japan after commercial blood banks were entirely abolished in 1969[14].

Chronic hepatitis C with cirrhosis is a strong risk factor for hepatocellular carcinoma (HCC)[15,16]. It has been shown that the risk of HCC increases with the degree of liver fibrosis[17]. Until recently, however, the degree of liver fibrosis could be reliably assessed only with liver biopsy, an invasive procedure with the possibility of serious complications[18,19].

Liver stiffness, which can be noninvasively measured with transient elastography, has been recently reported to be well correlated with histologically assessed liver fibrosis stage[20]. We previously reported that liver stiffness is strongly associated with the risk of HCC[21,22]. The calculated stratum-specific likelihood ratio indicated that the post-test odds for the presence of HCC increase five-fold when liver stiffness is higher than 25 kPa and decrease to one-fifth when lower than 10 kPa. Furthermore, we have confirmed in a prospective study that liver stiffness is a significant risk factor for HCC development, together with male gender, clinical cirrhosis and serum albumin level. However, in those studies we did not fully consider the duration of HCV infection and the age at the onset of infection, which are indicated in several studies to be associated with disease progression.

The aim of this study is to evaluate the association between liver stiffness and the duration of infection in blood transfusion-associated hepatitis C patients with and without HCC, focusing on the risk of HCC development.

MATERIALS AND METHODS

Patients

This study conformed to the ethical guideline of the 1975 Helsinki Declaration and the ethical guidelines for epidemiologic research designed by Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labor and Welfare. The study design was approved by the ethics committee of the authors' institution. Between December 2006 and June 2008, a total of 1562 patients positive for HCV RNA visited the liver clinic of authors' institution. Among these patients, those with a history of receiving blood transfusion were consecutively enrolled (229 with HCC and 295 without). We excluded from the present study those patients with concomitant hepatitis B virus surface antigen positivity, patients with uncontrollable ascites, patients on interferon therapy, and patients who received multiple blood transfusions.

Transient elastography

Liver stiffness was obtained noninvasively by using Fibroscan (Echosens, Paris, France), a newly developed medical device based on elastometry. Liver stiffness measurement (LSM) was considered valid only when at least eight acquisitions were successful with a success rate of at least 60% and the ratio of inter-quartile range (IQR) to the median value was larger than 30%.

Diagnosis of hepatocellular carcinoma

In patients with HCC, the cancer was diagnosed by dynamic computed tomography (CT), where intrahepatic nodules with hyperattenuation in the arterial phase with washout in the late phase were considered as definite HCC[23,24]. Histopathological diagnosis, using ultrasound-guided biopsy samples, was also performed when required. In patients without HCC, the cancer was ruled out by ultrasonography. No HCC was detected in the subsequent six-month follow-up period among these patients.

Laboratory tests

We determined the following parameters on the day of LSM: serum albumin and total bilirubin concentrations, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, prothrombin activity and platelet count. Serogrouping of HCV was assessed by enzyme-linked immunosorbent assay (ELISA) (Immucheck F-HCV Gr Kokusai; Sysmex, Kobe, Japan)[25]. Based on the prevalence of each HCV genotype in Japan, serogroup 1 was assumed to represent genotype 1b and serogroup 2 to represent genotype 2a or 2b.

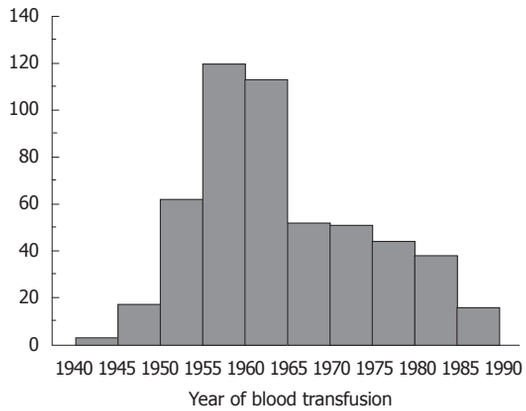

Figure 1 Frequency distribution of the year of receiving blood transfusion among the subjects. There is a peak around the year 1960.

Table 1 Characteristics of patients according to presence of hepatocellular carcinoma *n* (%)

Characteristics	HCC	Non-HCC	*P* value
Overall patients	*n* = 225	*n* = 291	
Gender (M/F)	126/99	118/173	0.0005
Age (yr)[1]	71.2 (66.1-75.7)	68.1 (58.7-72.4)	< 0.0001
Platelet count (10^9/L)[1]	95 (74-133)	161 (111-200)	< 0.0001
ALT (IU/L)[1]	48 (34-68)	42 (25-69)	0.006
Alcohol consumption > 50 g/d	51 (22.7)	28 (9.6)	< 0.0001
Liver stiffness (kPa)[1]	25.8 (17.3-37.4)	7.6 (5.6-13.9)	< 0.0001
IQR (kPa)[1]	4.0 (2.5-6.0)	1.6 (1.2-2.6)	< 0.0001
Duration (17-29 yr)	*n* = 34	*n* = 64	
Gender (M/F)	25/9	38/26	0.0028
Age (yr)[1]	73.1 (65.7-77.1)	59.7 (47.2-69.2)	0.033
Platelet count (10^9/L)[1]	95 (76-154)	180 (116-229)	< 0.0001
ALT (IU/L)[1]	51 (34-89)	42 (22-77)	0.2071
Alcohol consumption > 50 g/d	12 (35.3)	9 (14.1)	0.023
Liver stiffness (kPa)[1]	26.1 (16.8-53.3)	5.9 (4.9-12.1)	< 0.0001
Duration (30-39 yr)	*n* = 40	*n* = 59	
Gender (M/F)	16/24	23/36	0.9191
Age (yr)[1]	72.0 (65.4-76.7)	62.3 (55.7-68.6)	< 0.0001
Platelet count (10^9/L)[1]	93 (68-120)	151 (97-215)	< 0.0001
ALT (IU/L)[1]	42 (33-65)	48 (27-80)	0.7591
Alcohol consumption > 50 g/d	6 (15)	7 (11.9)	0.7641
Liver stiffness (kPa)[1]	28.7 (20.1-37.8)	7.4 (5.7-13.8)	< 0.0001
Duration (40-49 yr)	*n* = 101	*n* = 127	
Gender (M/F)	58/43	51/76	0.0113
Age (yr)[1]	69.2 (65.8-73.6)	69.9 (65.7-72.7)	0.8107
Platelet count (10^9/L)[1]	97 (67-136)	163 (112-195)	< 0.0001
ALT (IU/L)[1]	48 (34-69)	38 (23-64)	0.0080
Alcohol consumption > 50 g/d	25 (24.8)	8 (6.3)	0.0001
Liver stiffness (kPa)[1]	25.1 (17.5-37.4)	8.2 (5.8-14.1)	< 0.0001
Duration (50-70 yr)	*n* = 50	*n* = 41	
Gender (M/F)	27/23	18/23	0.4016
Age (yr)[1]	74.4 (70.0-78.1)	73.7 (66.3-79.2)	0.5658
Platelet count (10^9/L)[1]	97 (81-141)	147 (117-189)	0.0001
ALT (IU/L)[1]	52 (36-69)	46 (32-63)	0.1700
Alcohol consumption > 50 g/d	8 (16)	4 (9.8)	0.5363
Liver stiffness (kPa)[1]	16.0 (8.0-36.3)	7.9 (6.5-15.8)	< 0.0001

[1]Data are expressed as median (25th-75th percentiles). ALT: Alanine aminotransferase; IQR: Inter-quartile range; HCC: Hepatocellular carcinoma; M: Male; F: Female.

Duration of infection and liver stiffness progression

The date of blood transfusion was obtained by interview. Duration of infection was derived from the interval between the date of blood transfusion and the date of LSM. The rate of liver stiffness progression was calculated as follows: present liver stiffness-minimal stiffness value in the cohort (kPa)/interval after blood transfusion (years).

Statistical analysis

Data were expressed as median and 25th-75th percentiles in parenthesis unless otherwise indicated. The categorical variables were compared by χ^2 tests, whereas continuous variables were compared with unpaired Student's *t* test (parametric) or Mann-Whitney *U* test (non-parametric). A P value < 0.05 on two-tailed test was considered significant.

The correlation between liver stiffness and the interval from blood transfusion was assessed by Spearman's rank correlation. The duration of infection was arbitrarily stratified into 4 groups, 17-29 years; 30-39 years; 40-49 years; and 50-70 years. The difference in liver stiffness according to the presence of HCC was assessed in each group. Multiple linear regression analysis was used to determine the factors associated with liver stiffness. Processing and analysis were performed by using the S-plus Version 7 (TIBCO Software Inc., Palo Alto, CA, United States).

RESULTS

Patients' profile

A total of 524 patients underwent LSM. Eight patients were excluded because of unsuccessful measurements, mostly due to obesity (four patients had IQR/median > 30% and four had a success rate lower than 60%). Thus 516 patients were included in the current analysis (225 with HCC and 291 without). Their characteristics at the time of LSM are summarized in Table 1. The patients were 244 men and 272 women, with a mean age of 67.8 ± 9.5 years. The median liver stiffness was 14.3 kPa (25.8 in HCC group and 7.6 in non-HCC group). Figure 1 shows the frequency distribution of the year of receiving blood transfusion among the subjects. A peak is noted around the year 1960.

Characteristics of patients according to the duration of infection

Characteristics of patients were compared between patients with and without HCC in each duration of infection group (Table 1). The patients who developed HCC in short duration of infection were male dominant, having low platelet count, with a history of heavier alcohol consumption, showing higher liver stiffness, and receiving blood transfusion at an older age.

Correlation between liver stiffness and duration of infection

The correlation between liver stiffness and duration of

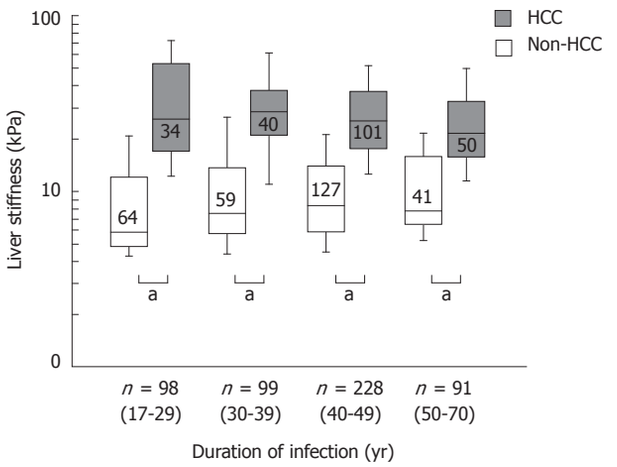

Figure 2 Duration of infection and liver stiffness. Liver stiffness was higher in patients with HCC than in patients without in each infection duration group ($^aP < 0.0001$ by Mann-Whitney *U* test).

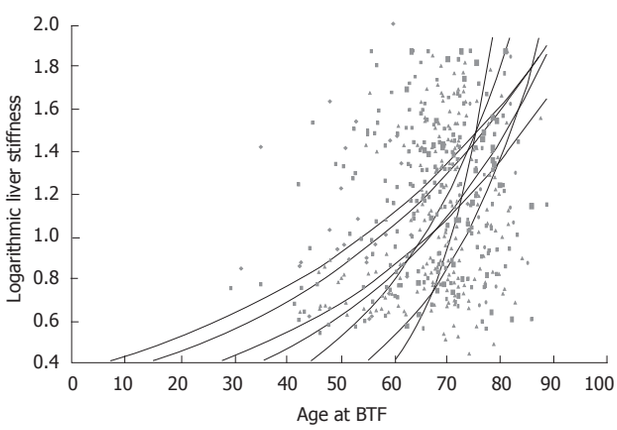

Figure 3 Age at blood transfusion and liver stiffness. Stiffness at present (each dot) and stiffness at BTF (assumed to normal value) were connected approximate logarithmic curve. Stiffness progressions become rapid in older age at BTF.

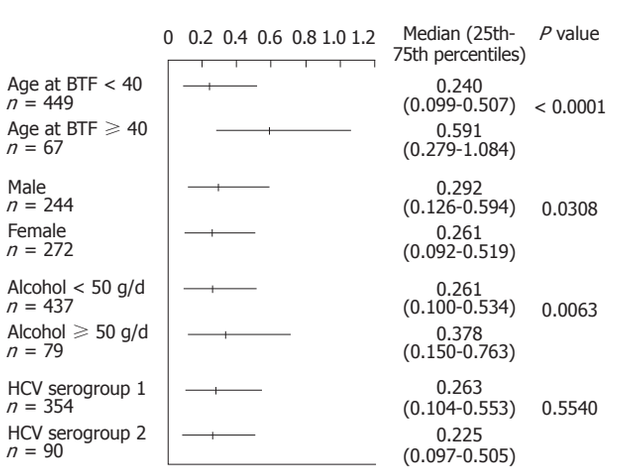

Figure 4 Liver stiffness progression rate. The progression rate is significantly higher in patients who were older than 40 at the time of blood transfusion, whose alcohol consumption is more than 50 g/d, and who are male. There is no significant difference according to hepatitis C virus (HCV) serotypes. Horizontal bar represents median value and 25th-75th percentiles.

infection was significant in patients without HCC ($r = 0.132$, $Z = 2.256$, $P = 0.024$) but not in patients with HCC ($r = -0.103$, $Z = -1.54$, $P = 0.123$). When the duration of infection was stratified into 4 groups, 17-29 years; 30-39 years; 40-49 years; and 50-70 years, liver stiffness was higher in patients with HCC than in patients without in each group ($P < 0.0001$, Figure 2).

Multiple regression analysis

The relationship between present liver stiffness and patients' characteristics, i.e., the age at blood transfusion, duration of infection, gender, and alcohol consumption (alcohol > 50 g/d) was analyzed with multiple linear regression analysis. The results showed that the age at blood transfusion was positively correlated with liver stiffness, with a coefficient of +0.336 per year for kPa, $P < 0.0001$, independently of the duration of infection (coefficient +0.272 per year for kPa, $P = 0.0015$). This suggests that fibrosis progression is more rapid when infection is acquired at older ages. Alcohol consumption was also significantly correlated with a positive coefficient (coefficient +4.183 for kPa, $P = 0.043$).

Stiffness progression and the age at blood transfusion

The progression of liver fibrosis, as represented by the increase in liver stiffness, must have been rapid in patients who have high liver stiffness in spite of short duration of HCV infection. We assumed that the liver stiffness was normal, that is, 2.9 kPa, when patients received blood transfusion. In Figure 3, the slopes represent the estimated increase rates of liver stiffness. In accordance with the results of multiple regression, the estimated increase rate was higher when patients received blood transfusion at older ages.

The progression rate of liver stiffness was assessed in subgroups according to three parameters (Figure 4). The progression rate was significantly higher in patients who were older than 40 at the time of blood transfusion ($P < 0.0001$), which is in accordance with the results of multiple regression. Heavy alcohol consumption (more than 50 g ethanol/d, $P = 0.0308$) and male gender ($P = 0.0063$) also showed significant difference by Mann-Whitney *U* test. There was no significant difference among HCV genotypes.

DISCUSSION

The natural history of chronic hepatitis C concerning liver fibrosis progression has been vigorously studied using liver biopsy specimens. The extent of liver fibrosis is usually evaluated as categorical stages. For example, METAVIR Score uses five stages, F0-F4, for fibrosis evaluation[26]. The fibrosis progression in hepatitis C patients, calculated by using paired liver biopsy, was reported to be 0.1-0.133 Unit per year[12,13]. Liver stiffness measured by transient elastography is now widely accepted as a surrogate marker of liver fibrosis[27]. Liver stiffness is expressed as a continuous variable in kPa unit. The cut-

off for cirrhosis is reportedly 13-17 kPa, and the upper limit of measurement is currently 75 kPa. Thus LSM has a wider dynamic range than histological staging, and the rate of fibrosis progression may be more accurately analyzed with LSM.

In the present study, the increase rate of liver stiffness was positively correlated with the age at blood transfusion, as shown by the steeper slopes of approximation curves when patients received BTF at older ages. The cause of this phenomenon is not clear but age-related changes in immunity may be involved. If this is the case, the increase rate is likely to become higher in the same patient with age. Indeed, each approximation curve in the figure apparently becomes steeper with age, suggesting age-related acceleration. This is to be confirmed in future longitudinal studies.

LSM is useful not only as a surrogate of liver biopsy but also as a risk indicator of HCC development. Indeed, in the present study, liver stiffness is high in patients with HCC regardless of duration of infection. The patients who developed HCC with short duration of infection received blood transfusion at an older age and were older at the time of LSM, male dominant, and showed higher liver stiffness than patients without HCC with similar duration of infection. The difference between patients with and without HCC became smaller with longer duration of infection, as the average liver stiffness in patients with HCC became lower and that in patients without HCC became higher. We speculated that patients with high liver stiffness who received blood transfusion in the early period have already died of HCC or liver failure and were eliminated from the study population. Another possibility is that HCC may develop in patients with relatively low liver stiffness when infection has lasted a long time.

In the present study, the median increase in liver stiffness was calculated as 0.275 kPa per year. Using 13.01 kPa as a cut-off for cirrhosis[28], it will take around 40 years on average to develop cirrhosis, which is consistent with previous reports based on liver biopsy[29]. Admittedly, the present study is basically cross-sectional, and prospective longitudinal LSM will be obviously superior in understanding the natural course of liver fibrosis progression. However, the estimated average increase rate of liver stiffness indicates that such studies will require repeated LSM at an interval of at least five years.

Age at viral infection, alcohol consumption, and male gender were reported to be associated with accelerated fibrosis progression[8-11]. In the present study, we performed subgroup analysis and indeed found that blood transfusion at an age older than 40, male gender, and alcohol consumption more than 50 g ethanol/d were significantly associated with rapid increase in liver stiffness. There is consensus that heavy alcohol consumption is associated with fibrosis progression[30]. Alcohol, which by itself can cause liver disease and fibrosis, may affect liver stiffness and worsen fibrosis in hepatitis C[31]. We did not find a difference in liver stiffness increase rate between HCV genotypes 1 (mostly 1b) and 2 (2a/2b), although we could not evaluate genotypes 1a, 3 or 4.

This study has some limitations. First, since this is a cross-sectional study performed after LSM became available, patients with more rapid disease progression may have died and been excluded from the study. Second, because transfusion-associated HCV infection has been virtually eliminated in Japan since 1992, we could not include patients with shorter duration of infection. Lastly, we did not perform paired LSM but assumed that liver stiffness was normal at the time of infection. Longitudinal observation is now on-going but will take several years to obtain results.

In conclusion, although liver stiffness gradually increases over time from the onset of infection in general, HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired HCV infection at older ages showed higher increase rate of liver stiffness and probably more rapid disease progression.

COMMENTS

Background

Liver stiffness, which can be noninvasively measured with transient elastography, has been recently reported to be well correlated with histologically assessed liver fibrosis stage.

Research frontiers

This study evaluated the association between liver stiffness and the duration of infection in blood transfusion-associated hepatitis C patients with and without hepatocellular carcinoma (HCC), focusing on the risk of HCC development.

Innovations and breakthroughs

Liver stiffness is expressed as a continuous variable in kPa unit. The cut-off for cirrhosis is reportedly 13-17 kPa, and the upper limit of measurement is currently 75 kPa. Thus liver stiffness measurement (LSM) has a wider dynamic range than histological staging, and the rate of fibrosis progression may be more accurately analyzed with LSM.

Applications

Although liver stiffness gradually increases over time from the onset of infection in general, HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired hepatitis C virus (HCV) infection at older ages showed higher increase rate of liver stiffness and probably more rapid disease progression.

Terminology

Transient elastography (Fibro-Scan®; EchoSens, Paris, France) is a rapid, reliable and well-tolerated imaging technique for the assessment of liver fibrosis by measuring liver stiffness.

Peer review

This is an interesting and timely study on liver stiffness in patients with transfusion associatated HCV. The authors show that HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired HCV infection at older ages showed higher increase rate of liver stiffness. Co-exposure to alcohol is critical. The methodology is sound and the paper is well and clearly written.

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BRIEF ARTICLE

Opiate-induced constipation related to activation of small intestine opioid μ2-receptors

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Abstract

AIM: To investigate the role of opioid μ-receptor subtype in opiate-induced constipation (OIC).

METHODS: The effect of loperamide on intestinal transit was investigated in mice. Ileum strips were isolated from 12-wk-old male BALB/c mice for identification of isometric tension. The ileum strips were precontracted with 1 μmol/L acetylcholine (ACh). Then, decrease in muscle tone (relaxation) was characterized after cumulative administration of 0.1-10 μmol/L loperamide into the organ bath, for a concentration-dependent study. Specific blockers or antagonists were used for pretreatment to compare the changes in loperamide-induced relaxation.

RESULTS: In addition to the delay in intestinal transit, loperamide produced a marked relaxation in isolated ileum precontracted with ACh, in a dose-dependent manner. This relaxation was abolished by cyprodime, a selective opioid μ-receptor antagonist, but not modified by naloxonazine at a dose sufficient to block opioid μ-1 receptors. Also, treatment with opioid μ-1 receptor agonist failed to modify the muscle tone. Moreover, the relaxation by loperamide was attenuated by glibenclamide at a dose sufficient to block ATP-sensitive K^+ (K_{ATP}) channels, and by protein kinase A (PKA) inhibitor, but was enhanced by an inhibitor of phosphodiesterase for cyclic adenosine monophosphate (cAMP).

CONCLUSION: Loperamide induces intestinal relaxation by activation of opioid μ-2 receptors via the cAMP-PKA pathway to open K_{ATP} channels, relates to OIC.

Key words: ATP-sensitive K^+ channels; Isometric tension; Loperamide; Opioid μ-receptors; Small intestine

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INTRODUCTION

Opiate-induced constipation (OIC) is widely observed among patients receiving chemotherapy[1,2]. In the gastrointestinal system, the opioid peptides are released and activate opioid receptors, which regulate the enteric circuitry by controlling motility and secretion, resulting in an increase in sphincter tone, inhibition of gastric emptying, and induction of stationary motor patterns. Together with the inhibition of ion and fluid secretion, these effects result in constipation, one of the most troublesome

side effects of opiate analgesic treatment[3]. The development of a better therapy for treating OIC is urgent and necessary.

Loperamide is widely used clinically to treat a variety of diarrheal syndromes, including acute and nonspecific (infectious) diarrhea[4,5]. Loperamide is a peripheral agonist of opioid μ-receptors with poor ability to penetrate the blood-brain barrier[6,7]. Some analgesic agents have been shown to have relaxant effects on smooth muscle[8,9]. (+)-Tramadol activates peripheral opioid μ-receptors, inducing concentration-dependent relaxation of the aorta[10]. Opioid μ-receptors are divided into three subtypes: μ-1, μ-2 and μ-3[11]. The activation of opioid μ-1 receptors has been reported to be associated primarily with the phospholipase C (PLC)-protein kinase C (PKC) pathway[12]. PLC-PKC signals can increase the intracellular calcium concentration, inducing gastrointestinal or bladder contraction[13,14]. Therefore, it is unlikely that intestinal relaxation is induced by the activation of opioid μ-1 receptors.

ATP-sensitive K^+ (K_{ATP}) channels are involved in the regulation of intestinal smooth muscle[15]. In addition, the opening of K_{ATP} channels has been reported to reduce intracellular Ca^+ concentration[16-18]. The K_{ATP} channel opener diazoxide has been shown to have the ability to attenuate indomethacin-induced small intestinal damage in rats[19]. However, the role of K_{ATP} channels in loperamide-induced gastrointestinal transit remains obscure.

In an attempt to determine the subtype of opioid μ-receptors involved in the regulation of intestinal tone, we used loperamide as an agonist to induce intestinal relaxation in the present study. In addition, specific blockers or antagonists were applied to investigate the potential mechanisms of action of loperamide.

MATERIALS AND METHODS

Experimental animals

We obtained 12-wk-old male BALB/c mice from the Animal Center of National Cheng Kung University Medical College. Mice were maintained in a temperature-controlled room (25 ± 1 ℃) under a 12-h light-dark cycle (lights on at 06:00 h). All mice were given water and fed standard chow (Purina Mills, LLC, St Louis, MO, United States) *ad libitum*. All animal-handling procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the Animal Welfare Act.

Gastrointestinal transit assay

Gastrointestinal tract (GIT) in mice was measured according to the method used in a previous study[20]. Briefly, 18 h before the experiment, food was withheld from the animals but free access to water was allowed. The mice received 0.25 mL of a suspension of charcoal consisting of 10% vegetable charcoal in 5% gum acacia (Sigma-Aldrich, St Louis, MO, United States) that was administered by an intragastric cannula. In subsequent experiments, the effects of loperamide or other compounds on GIT were evaluated 20 min after administration of the marker. At that time, the animals were sacrificed, the stomach and small intestine removed and the omentum was separated, avoiding stretching. The length of the intestine from the pyloric sphincter to the ileocecal junction and the distance travelled by the charcoal front were measured and recorded. We also recorded the time at which the mice started to drain stool after the administration of the charcoal meal.

We evaluated the effects of loperamide, stevioside (an agonist of opioid μ-1 receptor) or vehicle on GIT using subcutaneous injection into mice. Loperamide, stevioside and vehicle were given 30 min before the charcoal meal. The opioid antagonists cyprodime and naloxonazine were intraperitoneally injected at 60 min before the charcoal meal.

Preparation of isolated ileum

In the *in vitro* experiments, isolated ileum from BALB/c mice was used. Each mouse was killed by decapitation under anesthesia with pentobarbital (50 mg/kg). After the ileum strips had been carefully freed from the fat and connective tissue, the strips were mounted in organ baths filled with 10 mL oxygenated Krebs' buffer (95% O_2, 5% CO_2) at 37 ℃ containing: 135 mmol/L NaCl; 5 mmol/L KCl; 2.5 mmol/L $CaCl_2$; 1.3 mmol/L $MgSO_4$; 1.2 mmol/L KH_2PO_4; 20 mmol/L $NaHCO_3$; and 10 mmol/L D-glucose (pH 7.4). Each preparation was connected to strain gauges (FT03; Grass Instruments, Quincy, MA, United States). The isometric tension was recorded using chart software (MLS023, Powerlab; ADInstruments, Bella Vista, NSW, Australia). Strips were mounted and allowed to stabilize for 2 h. Each preparation was then gradually stretched to achieve an optimal resting tension of 0.5 g.

Intestinal relaxation induced by loperamide

After the resting tension had stabilized, a solution of acetylcholine (ACh; Sigma-Aldrich) prepared in distilled water was added to the bathing buffer to induce a rapid increase in ileum tone followed by stable constriction (tonic contraction). The final ACh concentration in the organ bath was 1 μmol/L. Ileum strips in the treatment group were exposed to loperamide (0.1-10 μmol/L) to observe the decrease in the tonic contraction (ileum relaxation). In addition, stevioside, an opioid μ-1 receptor agonist[21], was also used to investigate the effect on tonic contraction. Relaxation was expressed as the percentage decrease in the maximum tonic contraction. Concentration-relaxation curves were generated in a cumulative fashion.

Effects of antagonists on loperamide-induced intestinal relaxation

Ileum strips were exposed to glibenclamide (Research Biochemicals, Wayland, MA, United States), a specific opioid μ-1 receptor antagonist (naloxonazine) or a general opioid μ receptor antagonist (cyprodime) (Tocris Cookson, Bristol, United Kingdom), for 15 min before addition of loperamide to the organ bath. The strips were treated with an inhibitor of cyclic adenosine monophosphate (cAMP) phosphodiesterase (3-isobutyl-1-methylxanthine; IBMX) or an inhibitor of protein kinase A (PKA) (H-89)

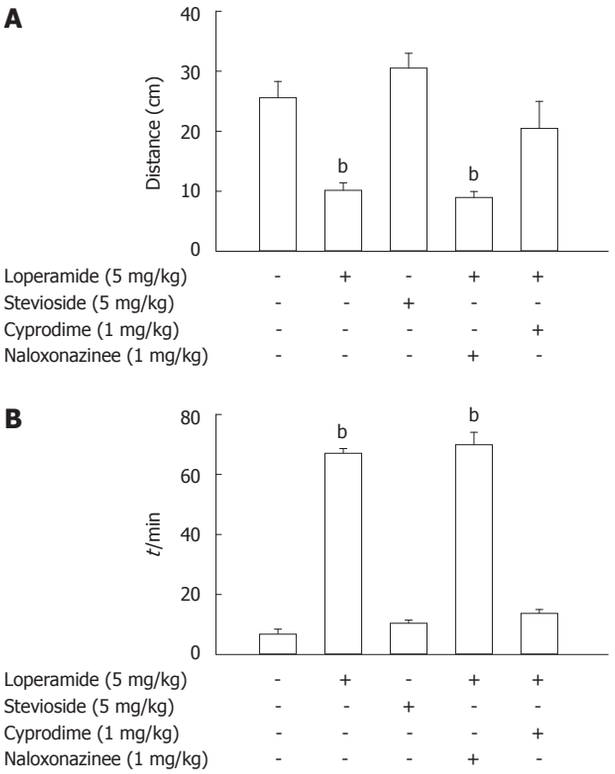

Figure 1 Role of opioid μ-receptors in gastrointestinal tract using charcoal as an indicator. The data represent the distance (A) and time (B) for the transit of charcoal. Data represent the mean ± SEM of eight animals. $^{b}P < 0.01$ *vs* the distilled water (vehicle)-treated control.

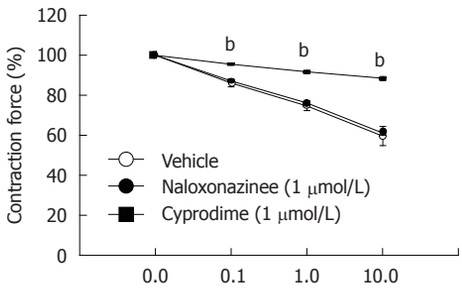

Figure 2 Inhibitory effect of cyprodime or naloxonazine on relaxation induced by loperamide (10 μmol/L) in isolated ileum contracted with 1 μmol/L acetylcholine. Data represent the mean ± SEM of the percentage changes in the acetylcholine (ACh)-induced tonic contraction of ileum from eight animals. $^{b}P < 0.01$ *vs* the distilled water (vehicle)-treated control.

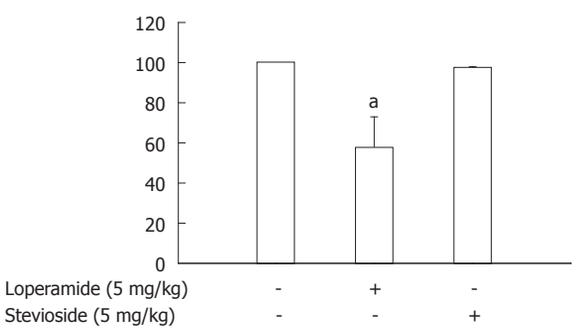

Figure 3 Effect of stevioside on the tone of isolated ileum strips contracted with 1 μmol/L acetylcholine. Data represent the mean ± SEM of the percentage changes in the acetylcholine (ACh)-induced tonic contraction of ileum from eight animals. $^{a}P < 0.05$ *vs* the distilled water (vehicle)-treated control, shown in the first column.

in the same manner. Forskolin (Sigma-Aldrich) was used as a control. The changes in the relaxation caused by antagonists or blockers were compared with those of the vehicle-treated control.

Statistical analysis

All values are presented as the mean ± SEM for a given number of animals or samples. Analysis of variance and Dunnett's post hoc test were used to evaluate the significance between groups. $P < 0.05$ was considered significant.

RESULTS

Role of opioid receptor in loperamide-induced gastrointestinal transit

As shown in Figure 1A, the distance travelled by charcoal in the loperamide-treated group (5 mg/kg) was shorter than that in the vehicle-treated group. However, the distance travelled in the stevioside-treated (5 mg/kg) group was similar to that in the vehicle-treated group. In addition, pretreatment with cyprodime (1 mg/kg) significantly abolished the effect of loperamide on GIT, but naloxonazine (1 mg/kg) failed to produce the same effect. Moreover, the time for transit of charcoal from the stomach to the anus stool drain in the loperamide-treated group (5 mg/kg) was longer than that in the vehicle-treated group. The transit time of the stevioside-treated (5 mg/kg) group was the same as that of the vehicle-treated group. In addition, pretreatment with cyprodime (1 mg/kg) attenuated the loperamide-induced delay in charcoal transit, but naloxonazine (1 mg/kg) failed to exhibit the same action (Figure 1B).

Effect of opioid receptor blockade on loperamide-induced intestinal relaxation

Ileum strips strongly contracted in response to the application of ACh at 1 μmol/L. As shown in Figure 2, loperamide relaxed the ACh-contracted ileum strips in a concentration-dependent manner. At the maximum concentration tested (10 μmol/L), loperamide significantly attenuated the tonic contraction of ileum strips to 63.59% ± 5.60% of the contraction induced by ACh. Cyprodime (1 μmol/L) produced a marked attenuation of the relaxation induced by loperamide. However, naloxonazine failed to modify the action of loperamide, even at a higher concentration (1 μmol/L). In addition, treatment with stevioside at a dose sufficient to activate the opioid μ-1 receptor[21] failed to modify the intestinal tone in ACh-contracted ileum (Figure 3).

Role of cyclic adenosine monophosphate and protein kinase A in loperamide-induced intestinal relaxation

In the present study, forskolin (5 μmol/L), a direct activator of adenylate cyclase, was used as a positive control to increase the activity of cAMP, based on the findings of a previous study[22]. In ileum strips contracted with

Table 1 Effects of inhibitors of cyclic adenosine monophosphate-phosphodiesterase or protein kinase A on the relaxation

	ACh (%)
Loperamide (10 μmol/L)	
+ Vehicle	63.59 ± 5.60
+ H-89 (1 μmol/L)	80.49 ± 3.07[a]
+ IBMX (10 μmol/L)	41.02 ± 2.57[b]
+ Glibenclamide (1 μmol/L)	83.52 ± 0.89[a]
Forskolin (5 μmol/L)	
+ Vehicle	31.64 ± 7.39
+ H-89 (1 μmol/L)	79.29 ± 2.76[b]
+ IBMX (10 μmol/L)	27.77 ± 1.40[b]
+ Glibenclamide (1 μmol/L)	80.98 ± 2.75[b]
IBMX (10 μmol/L)	93.41 ± 2.15[b]
H-89 (1 μmol/L)	91.31 ± 3.47[b]
Glibenclamide (1 μmol/L)	92.45 ± 3.29[b]

Effects of inhibitors of cyclic adenosine monophosphate-phosphodiesterase or protein kinase A on the relaxation induced by loperamide (10 μmol/L) or forskolin (5 μmol/L) in isolated ileum contracted with 1 μmol/L acetylcholine (ACh). IBMX: 3-isobutyl-1-methylxanthine. [a]$P < 0.05$, [b]$P < 0.01$ *vs* vehicle treated control.

ACh (1 μmol/L), forskolin-induced relaxation was also abolished by pretreatment with glibenclamide (1 μmol/L). Moreover, intestinal relaxation induced by forskolin was increased by the addition of IBMX at a concentration (10 μmol/L) sufficient to inhibit cAMP-phosphodiesterase[23], and was decreased by addition of H-89 at a concentration (1 μmol/L), which was sufficient to inhibit the activity of PKA[24]. The loperamide-induced intestinal relaxation was also modified by these agents in the same manner. Our results showed that intestinal relaxation induced by loperamide was increased by IBMX and attenuated by H-89 (Table 1).

DISCUSSION

In the present study, we found that loperamide caused a dose-dependent delay in GIT using charcoal as an indicator in mice. In addition, loperamide induced relaxation in the ileum strips contracted with stimulant. This action of loperamide seems to be related primarily to the activation of opioid receptors in peripheral tissue, because loperamide does not cross into the central nervous system[7]. The loperamide-induced action was effectively abolished by cyprodime, suggesting that opioid μ receptors were involved. However, this action of loperamide was not reversed by naloxonazine even at a dose sufficient to block opioid μ-1 receptors. In addition, as shown in Figure 2, relaxation was not induced by stevioside, which is an agonist specific for opioid μ-1 receptors[21]. The involvement of opioid μ-1 receptors in the intestinal relaxation mechanism of loperamide seems unlikely.

Thus, another opioid μ receptor must be involved in this action of loperamide. There is no doubt that loperamide is an agonist of peripheral opioid μ receptors[7,25]. Opioid μ receptors have been divided into three subtypes[11]: μ-1, μ-2 and μ-3[26-28]. The analgesic action mediated by the activation of opioid μ-1 receptors has been reported to exert spinal antinociception[29,30]. In addition, the activation of opioid μ-1 receptors seems to be related to smooth muscle contraction *via* the PLC-PKC pathway[14,31]. Moreover, opioid μ-3 receptors are present predominantly in endothelial cells associated with the production of nitric oxide to induce vasodilatation[32]. Therefore, the involvement of opioid μ-1 or μ-3 receptors in intestinal relaxation seems unlikely. Taken together, our results suggest that the activation of opioid μ-2 receptors is more likely to participate in the action of loperamide with respect to intestinal relaxation. The activation of opioid μ-2 receptors has been reported to be involved in the relaxation of guinea pig ileum and in the inhibition of GIT[33,34]. In addition, opioid μ-receptor-expressing myenteric neurons are distributed primarily in the small intestine, followed by the stomach and the proximal colon[35]. Although constipation is predominantly a large bowel disorder, the presence of opioid μ receptors in the colon and the longer GIT time of charcoal to the anus stool drain in mice that received loperamide support the role of opioid μ-2 receptors in opiate-induced constipation. Unfortunately, there is no suitable tool or agent that can be used to provide further evidence supporting this hypothesis. Therefore, we focused on the subcellular signals as an alternative experimental approach.

Potassium channels play an important role in the regulation of intestinal smooth muscle cells[36,37]. ATP-sensitive K^+ (K_{ATP}) channels are composed of four inwardly rectifying K^+ channel subunits and four regulatory sulfonylurea receptors[38]. The activation of K_{ATP} channels induces hyperpolarization of the cell membrane and consequently relaxes the smooth muscle. Thus, we focused on the involvement of K_{ATP} channels in the intestinal relaxation induced by loperamide. We used forskolin as a positive control because forskolin is a direct activator of adenylate cyclase that can increase intracellular cAMP concentration to activate cAMP-dependent PKA, resulting in the opening of K_{ATP} channels[24]. As shown in Table 1, we observed that forskolin-induced intestinal relaxation was also blocked by glibenclamide. The intestinal relaxation induced by forskolin was abolished by H-89 at a concentration sufficient to block the activity of PKA[24] and was enhanced by IBMX at a concentration sufficient to inhibit the activity of cAMP-posphodiesterase[23]. Similar changes were also observed in ileum strips relaxed by loperamide (Table 1). These data suggest that the potential mechanism responsible for loperamide-induced intestinal relaxation is mediated *via* the cAMP-PKA pathway to open K_{ATP} channels. Therefore, the results provide a novel insight into the mechanism of action of loperamide and increase our understanding of intestinal relaxation. It is reasonable to consider that similar results will be obtained for the parts of the colon that have opioid μ receptors. Further investigations are required in the future.

In conclusion, we suggest that the activation of opioid μ-2 receptors, which induce the opening of K_{ATP} channels, is responsible for loperamide-induced intestinal relaxation. Therefore, peripheral opioid μ-2 receptors will

be a new target in the development of agents for treating OIC.

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COMMENTS

Background

Opioid-induced constipation (OIC) is a frequent disorder in tumor patients receiving morphine-like compounds. Thus, it is important to prevent this disorder. To date, it is still unclear which subtype of opioid receptors should be used for development of suitable agents. Loperamide is a well-known agonist of opioid receptors, without the ability to enter the brain. Many studies have reported that loperamide can be used to treat diarrhea, but the receptor site has not been established.

Research frontiers

Loperamide is a widely used agent in clinics and its effectiveness is believed to arise from peripheral action. In the area of prevention of constipation with loperamide, the research hotspot is how to distinguish the receptor subtype to improve its adverse reactions. Then, it will be useful for prevention of OIC.

Innovations and breakthroughs

In previous studies of loperamide for treatment of diarrhea, it was found that intestinal motility was significantly decreased. In order to decrease the side effects of loperamide, the authors investigated the receptor subtype that is selectively activated, and the results will be useful for the development of new agents with similar side effects. Thus, the authors compared loperamide with stevioside, which is mainly effective against opioid μ-1 receptors. We found that opioid μ-2 receptors linked with ATP-sensitive K^+ channels are responsible for intestinal relaxation. Therefore, agents with less effect on opioid μ-2 receptors will be useful to decrease the side effects of constipation.

Applications

The results suggest that opioid μ-2 receptors are mainly responsible for intestinal relaxation. Clinical application of agents showing less affinity than loperamide to opioid μ-2 receptors could be useful for prevention of constipation.

Terminology

OIC is a frequent side effect in cancer patients who received morphine-like compounds to reduce pain. Loperamide is a widely used agent for treatment of diarrhea in clinics, and it has no effect in the brain. Opioid receptors are the action site of opioids and related agents. Receptors are generally expressed in various tissues and located on the cell membrane. Subtypes of opioid receptors have been established.

Peer review

This is a good descriptive study in which the authors analyzed the subtype of opioid μ receptors in the intestine of mice. The results are interesting and suggest that opioid μ-2 receptors are responsible for intestinal relaxation, which could be useful in preventing constipation by agents with less affinity for this receptor site.

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BRIEF ARTICLE

Notch3 regulates the activation of hepatic stellate cells

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Abstract

AIM: To investigate whether Notch signaling is involved in liver fibrosis by regulating the activation of hepatic stellate cells (HSCs).

METHODS: Immunohistochemistry was used to detect the expression of Notch3 in fibrotic liver tissues of patients with chronic active hepatitis. The expression of Notch3 in HSC-T6 cells treated or not with transforming growth factor (TGF)-β1 was analyzed by immunofluorescence staining. The expression of Notch3 and myofibroblastic marker α-smooth muscle actin (α-SMA) and collagen I in HSC-T6 cells transfected with pcDNA3.1-N3ICD or control vector were detected by Western blotting and immunofluorescence staining. Moreover, effects of Notch3 knockdown in HSC-T6 by Notch3 siRNA were investigated by Western blotting and immunofluorescence staining.

RESULTS: The expression of Notch3 was significantly up-regulated in fibrotic liver tissues of patients with chronic active hepatitis, but not detected in normal liver tissues. Active Notch signaling was found in HSC-T6 cells. TGF-β1 treatment led to up-regulation of Notch3 expression in HSC-T6 cells, and over-expression of Notch3 increased the expression of α-SMA and collagen I in HSC-T6 without TGF-β1 treatment. Interestingly, transient knockdown of Notch3 decreased the expression of myofibroblastic marker and antagonized TGF-β1-induced expression of α-SMA and collagen I in HSC-T6.

CONCLUSION: Notch3 may regulate the activation of HSCs, and the selective interruption of Notch3 may provide an anti-fibrotic strategy in hepatic fibrosis.

Key words: Notch signaling; Myofibroblast; Liver fibrosis; Hepatic stellate cells; siRNA

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INTRODUCTION

Hepatic fibrosis is a reversible wound-healing response characterized by the accumulation of extracellular matrix (ECM) to liver injury[1]. In the process of hepatic fibrosis, activated hepatic stellate cells (HSCs) synthesize a large amount of ECM and then change into myofibroblasts[2], which is characterized by the expression of α-smooth

muscle actin (α-SMA) and ECM, particularly collagen I. Myofibroblast is one of the key cellular components involved in liver fibrosis, therefore, the majority of anti-fibrotic therapies are designed to inhibit the activation, proliferation, or synthetic products of HSCs[3].

Notch signaling is an ancient cell signaling that regulates cell fate specification, stem cell maintenance, and initiation of differentiation in embryonic and postnatal tissues[4,5]. More recently, some researches reported that Notch signaling was implicated in human fibrosis diseases, such as pulmonary, renal and peritoneal fibrosis[6-8]. Several researches suggested that the Jagged/Notch pathway may selectively mediate fibrogenic properties of transforming growth factor-β1 (TGF-β1) which was essential to promote the production and deposition of ECM[9-11].

Notch receptors (Notch1, Notch2 and Notch4) were present at the mRNA level in freshly isolated HSCs, and the synthesis of Notch1 decreased during culture and development of HSCs into myofibroblast-like cells[12]. However, the expression of Notch3 in phenotype activated HSCs remains unknown. Ono *et al*[13] reported that Notch3 was required for TGF-β1-induced myofibroblastic differentiation of myoblasts. Based on the studies above, the present study was undertaken to investigate whether Notch3 was expressed in fibrotic liver tissues of patients with chronic active hepatitis and in activated HSCs, and sequentially contributed to liver fibrosis by regulating the activation of HSCs.

MATERIALS AND METHODS

Patients and liver biopsy samples

Liver tissue samples were obtained by biopsy from 11 patients with chronic active hepatitis (5 women and 6 men; median age 43 years, range 31-55 years). Control liver biopsy specimens were obtained from healthy volunteers (n = 6). All patients and controls signed consent forms approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Tissue samples were fixed in 10% formalin and paraffin-embedded for immunohistochemical analysis.

Antibodies and reagents

Rabbit polyclonal to α-SMA and Notch3 were obtained from Abcam (Cambridge, United States). Rabbit polyclonal anti-collagen I antibody was purchased from Bioss Corporation (Beijing, China). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, United States). Recombinant human transforming growth factor (TGF)-β1 was purchased from PeproTech EC Ltd (London, United Kingdom). Lipofectamine™ 2000 transfection reagent was obtained from Invitrogen (Carlsbad, United States).

Cell line and culture conditions

HSC-T6 cells, an immortalized rat HSC line, purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (China), were cultured in Dulbecco's -modified Eagle's medium (DMEM; Gibco, United States) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (Gibco, United States). TGF-β1 (2 ng/mL) was incubated in growth medium for 24 h.

Immunohistochemistry and immunocytofluorescence analysis

Liver tissue sections were incubated with 3% H_2O_2 followed by serum blocking with 10% goat serum in 5% bovine serum albumin (BSA). The Notch3 was detected by staining with polyclonal rabbit anti-Notch3 (1:250) overnight at 4 ℃. Irrelevant isotype antibodies (Santa Cruz Biotechnology, United States) at the same concentration were used as control. The staining was carried out using SABC kit (Boster, China).

Cells were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde at room temperature for 30 min and were penetrated in blocking solution (Amresco, United States) containing 0.3% Triton X-100, and incubated overnight with either anti-Notch3 (1:250), anti-α-SMA (1:100) or anti-collagen I (1:100) antibody in 1% BSA solution. Then, cells were incubated with secondary antibodies for 1 h at 37 ℃. After incubation, cells were stained with nuclear stain marker 4',6-diamidino-2-phenylindole. The reaction was examined under confocal microscope (Nikon, Japan).

Transfection of siRNA and plasmid

HSC-T6 was seeded into 6-well plates at a density of 1×10^5 cells 12 h before transfection. siRNA was mixed with 5 μL lipofectamine 2000 in 250 μL Opti-MEM I medium for 20 min. The transfection mixture was then added to each well with 1.5 mL FBS free DMEM at a concentration of 100 nmol/L. After 6-h incubation, liquid mixture containing siRNA was disposed. Two mL DMEM containing 10% FBS was added and incubated for another 72 h. The following siRNA sequences were used: Notch3 siRNA: 5' ACAAGAUCAAUACAGGAGCTT 3'; the control siRNA sequence: 5' UUGUAC UACACAAAAGUACUG 3'. These siRNA were synthesized by Shanghai Genepharma Co. Ltd. (Shanghai, China).

HSC-T6 was transfected with Notch3 intracellular domain (Notch3-ICD) cDNA cloned into pcDNA3.1 (pcDNA3.1-N3ICD) vector, which was a gift from Dr. Tao Wan. The control (pcDNA3.1-empty) vector was purchased from Shanghai Genepharma Co. Ltd. (Shanghai, China). All transfections were performed using Lipofectamine 2000 following the manufacturer's instructions.

TaqMan quantitive reverse transcription polymerase chain reaction

Total RNA from each sample was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was performed using a StepOne/StepOne-Plus (ABI) and the TaqMan PCR Reagent (Genepharma). Primer sequences are summarized in Table 1. Comparative threshold (Ct)

Table 1 Primer sequences for TaqMan real-time reverse transcription polymerase chain reaction

Notch3	
Forward	5′-CCTGCCTGCCTCTATGACAAC-3′
Reverse	5′-ACACTCCTCGGTGTTACAGCC-3′
Probe	5′-ACTGCTACTCTGGTGGCCGCGAC-3′
Jagged1	
Forward	5′-GTGGAAGAGGATGATATGGATAAGC-3′
Reverse	5′-CTCCTCTCTGTCTACCAGCGTGTAC-3′
Probe	5′-CCAGCAGAAAGTCCGGTTTGCCA-3′
Hes1	
Forward	5′-TGCTACCCCAGCCAGTGTC-3′
Reverse	5′-GCTTTGATGACTTTCTGTGCTCA-3′
Probe	5′-CTGTCTTTGGTTTGTCCGGTGTCGT-3′
GAPDH	
Forward	5′-GATGACATCAAGAAGGTGGTGAAG-3′
Reverse	5′-ACCCTGTTGCTGTAGCCATATTC-3′
Probe	5′-ACTTCAACAGCAACTCCCACTCTTCCACC-3′

method was used for calculating the relative amount of mRNA of treated sample compared with control samples.

Western blotting assay

Cells were washed with PBS and lysed. The extracts were cleared by centrifugation at 12 000 × *g* for 15 min. After blocking with 5% non-fat milk in PBS containing 0.1% Tween 20 for 1 h at room temperature, membranes were incubated with either anti-Notch3 (1:1000), anti-α-SMA (1:300) or anti-collagen I (1:200) antibody in tris-buffered saline (TBS) containing 0.05% Tween 20 at 4 ℃ overnight. Then membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (1:2000) antibody in TBS and Tween 20 for 1 h at room temperature, and visualized by chemiluminescence using an electrochemiluminescence immunoblotting kit (Cell Signaling Technology) with a digital luminescent image analyzer BioSpectrum600 (UVP, United States). Band intensity was assessed using Gel-Pro analyzer.

Statistical analysis

All experiments were repeated three times, and data recorded as mean ± SD and analyzed by Student's *t* test using SPSS12.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Notch3 in fibrotic liver tissues of patients with chronic active hepatitis

All patients were positive for the Notch3 in fibrotic liver tissues (Figure 1A). In contrast, Notch3 was not detected by immunohistochemistry in normal liver tissues (Figure 1C).

Expression of Notch3 in hepatic stellate cell-T6 cells

To detect expression of Notch3 in HSCs, immunofluorescence staining analysis was performed to examine the expression of Notch3 in HSC-T6 cells. The result showed that Notch3 protein was localized in the cytoplasm and nucleus of HSC-T6 cells (Figure 2).

Up-regulation of Notch3 expression by transforming growth factor-β1 in hepatic stellate cell-T6 cells

We investigated the effect of TGF-β1 on Notch signaling in HSC-T6 treated with TGF-β1 (0.5, 1, 2 and 4 ng/mL) for 24 h. RT-PCR analysis showed that the expression of Notch signaling components including Notch3, Jagged1 and Hes-1 were obviously increased in HSC-T6 treated with 2 ng/mL TGF-β1 as compared with the control group without TGF-β1 treatment ($P < 0.05$, Figure 3).

Over-expression of Notch3 increased the expression of myofibroblastic marker in hepatic stellate cell-T6

To investigate the effect of Notch3 in activation of HSCs, we examined if overexpresion of Notch3 in HSC-T6 would enhance the activation. The results showed that the increased expression of Notch3 in pcDNA3.1-N3ICD introduced HSC-T6 cells as compared with cells transfected with pcDNA3.1-empty vector ($P < 0.05$, Figure 4A). Western blotting and immunofluorescence staining analyses demonstrated that over-expression of Notch3 led to increased expression of α-SMA and collagen I compared with control group ($P < 0.05$, Figure 4A and B).

Knockdown of Notch3 by siRNA downregulated the expression of myofibroblastic marker in hepatic stellate cell-T6

To further confirm the role of Notch3 in regulating activation of HSCs, siRNA was employed to specifically knockdown Notch3. Western blotting analysis showed that siRNAs targeting Notch3 reduced Notch3 protein levels by approximately 80%. Seventy and two hour after transfection in HSC-T6 ($P < 0.05$, Figure 5A). We also observed that knockdown of Notch3 in HSC-T6 down-regulated the expression of α-SMA and collagen I detected by Western blotting and immunofluorescence staining 72 h after siRNA transfection ($P < 0.05$, Figure 5A and B).

To investigate the relationship between Notch3 and TGF-β1, TGF-β1 (2 ng/mL) was added into HSC-T6 24 h before transfection with siRNAs targeting Notch3 or control siRNAs. Western blotting and immunofluorescence staining analyses demonstrated that knockdown of Notch3 antagonized TGF-β1-induced expression of α-SMA and collagen I in HSC-T6 ($P < 0.05$, Figure 5A and B).

DISCUSSION

Liver fibrosis is the result of the wound-healing response to repeated injury in liver. It is well known that HSCs activation plays an important role in fibrosis because these cells become the primary source of extracellular matrix in liver upon injury. TGF-β1 is known to promote fibrogenesis *in vivo* and *in vitro*, however, development of anti-fibrotic strategies targeting the TGF-β axis is problematic owing to the pleiotropic nature of TGF-β action[1].

Notch signaling is an evolutionarily conserved local cell-signaling that functions in the determination of cellular identity during developmental stages[14]. Four Notch proteins (Notch1, Notch2, Notch3 and Notch4) have

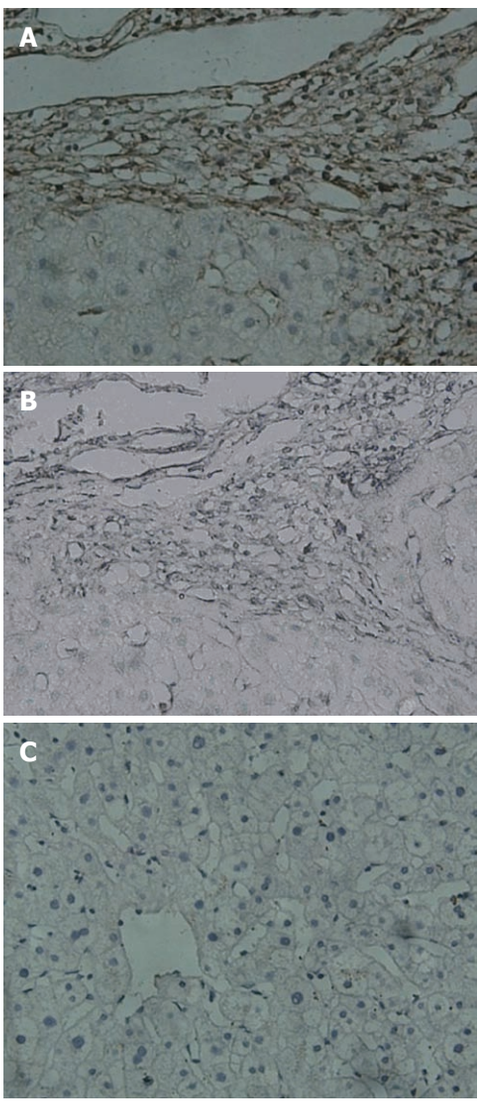

Figure 1 Immunohistochemical staining of Notch3 in liver tissues of patients with chronic active hepatitis and normal livers. A: Intense staining of Notch3 in fibrotic tissues of livers from patients with chronic active hepatitis; B: Negative control; C: Notch3 was not detected in normal liver tissues (x 400).

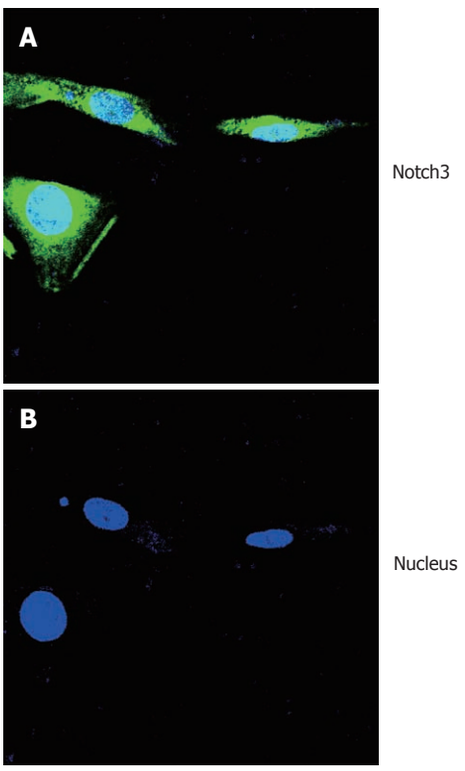

Figure 2 Immunofluorescence staining analysis was performed to examine expression of Notch3 in hepatic stellate cell-T6 cells. A: The green fluorescence represents the expression of Notch3; B: Blue one represents nucleus of hepatic stellate cell-T6 cells (x 400).

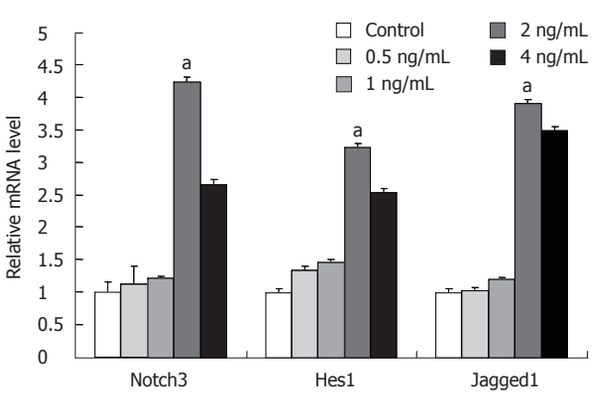

Figure 3 TaqMan reverse transcription polymerase chain reaction analysis was performed to detect expression of Notch3, Jagged1 and Hes1 in hepatic stellate cell-T6 cells treated or not with transforming growth factor-β1 (0.5, 1, 2 and 4 ng/mL) for 24 h. $^{a}P < 0.05$ *vs* control group.

been identified in vertebrates, while membrane-bound proteins (Delta and Jagged) have been recognized as Notch ligands[15]. Activation of the pathway usually occurs via expression of the ligand in signal-giving cells. Upon interaction with the ligand, Notch undergoes a series of proteolytic cleavages in the signal-receiving cells. Finally, the cytoplasmic domain, referred to as the Notch intracellular domain (NICD), translocates to the nucleus, where it binds with the transcription factor CSL [CBF-1/RBP-Jk, Su (H) and Lag-1] and co-activator Mastermind-like to trigger downstream target genes expression, such as HES1 and HEY which act as transcription factors[16-20].

Notch signaling is essential for the regulation of cell differentiation, and its aberrant activation was implicated in human fibrosis diseases. Notch1 signaling in response to inflammatory zone 1 may play a significant role in myofibroblast differentiation during lung fibrosis[6]. Active Notch pathway in tubular epithelial cells was demonstrated as a critical regulator of tubulointerstitial fibrosis[7]. It was reported that Notch signaling was highly activated in rats with fibrotic peritoneum induced by peritoneal dialysis fluid , as indicated by increased expression of Jagged1, Notch1, and HES1. Blocking Notch signaling activation by intraperitoneal injection of a γ-secretase inhibitor significantly attenuated peritoneal fibrosis as indicated by the decreased expression of α-smooth muscle actin and collagen I [8].

In this study, the Notch3 was not detected in normal liver tissues. In contrast, intense staining of the Notch3

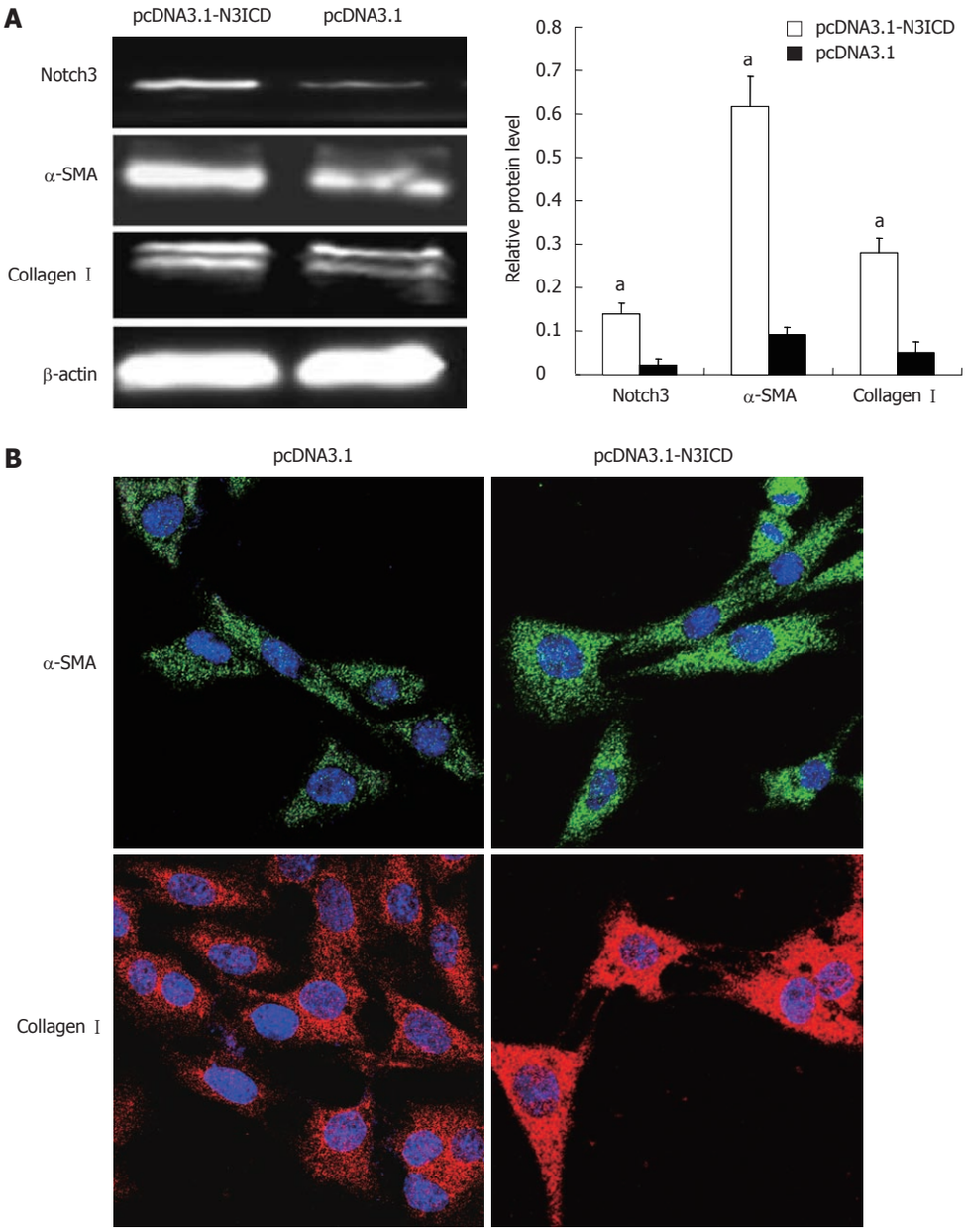

Figure 4 Over-expression of Notch3 intracellular domain increased the expression of myofibroblastic marker in hepatic stellate cell-T6. A: The expression of Notch3, α-smooth muscle actin (SMA) and collagen Ⅰ were detected by Western blotting. Graphic representation of relative level of Notch3, α-SMA and collagen Ⅰ normalized to β-actin; B: The expression of α-SMA and collagen Ⅰ was also detected by immunofluorescence staining (x 400), $^{a}P < 0.05$ *vs* control group.

was observed in fibrotic liver tissues of patients with chronic active hepatitis, which suggested that Notch3 was involved in liver fibrosis. Furthermore, several lines of evidence clearly confirmed that Notch3 contributed to liver fibrosis by regulating activation of HSCs. First, the expression of active Notch3 was found in the nucleus of HSC-T6. Second, the expression level of Notch signaling components was elevated including Notch3, Jagged1 and Hes1 in HSC-T6 after TGF-β1 treatment. More importantly, we also found that specific knockdown of Notch3 by siRNA antagonized TGF-β1-induced expression of myofibroblastic marker α-SMA and collagen I in HSC-T6, and on the contrary, over-expression of Notch3 increased the expression of myofibroblastic marker in HSC-T6.

Inhibition of the γ-secretase complex required for release of the active NICD is the most common method for targeting Notch signaling[21,22]. Two different inhibitors are being evaluated in clinical trials for the treatment of resistant T cell acute leukemia and advanced breast cancer (www.clinicaltrials.gov). However, targeting of γ-secretase would result in several adverse events when Notch receptors from Notch1 to Notch4 are affected[23,24]. In this study, we found that the selective interruption of Notch3 by siRNA decreased the expression of myofibroblastic marker in HSC-T6 cells, which provided a potential novel therapeutic target for liver fibrosis.

However, further studies are required to elucidate how do other members of Notch family, such as Notch1, Notch2 and Notch4, play a part in activated HSCs as

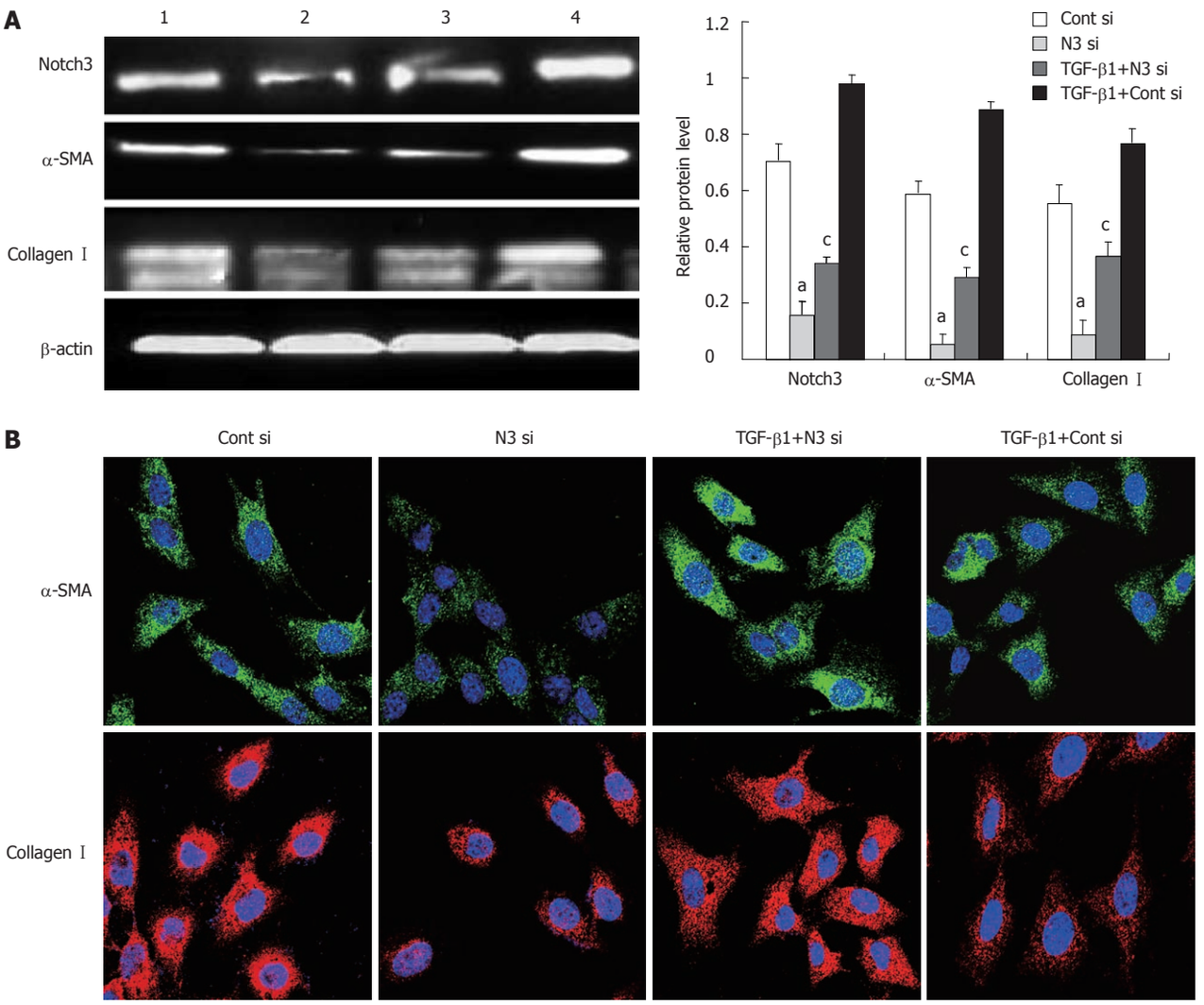

Figure 5 Knockdown of Notch3 decreased the secretion of myofibroblastic marker in hepatic stellate cell-T6. A: Effects of Notch3 knockdown in HSC-T6 using Notch3 siRNA (N3 si), and control siRNA (Cont si) were investigated. Expression of Notch3, α-smooth muscle actin (SMA) and collagen Ⅰ was detected by Western blotting. Graphic representation of relative level of Notch3, α-SMA and collagen Ⅰ normalized to β-actin; B: The expression of α-SMA and collagen Ⅰ was also detected by immunofluorescence staining (x 400). $^{a}P < 0.05$ *vs* control siRNA group, $^{c}P < 0.05$ *vs* transforming growth factor (TGF)-β1+control siRNA group. 1: Cont si; 2: N3 si; 3: TGF-β1+N3 si; 4: TGF-β1+Cont si.

well as the mechanism underlying the Notch signaling in liver fibrosis. In addition, the contribution of Notch and TGF-β1 signaling to the liver fibrosis should also be further investigated.

In conclusion, we demonstrated for the first time that Notch3 plays a role in regulating the activation of HSCs (HSC-T6). Therefore, the selective interruption of Notch3 may have a potential anti-fibrogenic effect in liver fibrosis.

COMMENTS

Background

It is well known that hepatic stellate cell (HSC) activation plays an important role in fibrosis because these cells are the primary source of extracellular matrix in liver upon injury. Notch signaling regulates many aspects of morphogenesis through diverse effects on differentiation, proliferation, and cell survival. Recently, some researches reported that Notch signaling was implicated in human fibrosis diseases, such as pulmonary, renal and peritoneal fibrosis.

Research frontiers

This study was undertaken to investigate whether Notch signaling is activated in HSCs and sequentially contributed to liver fibrosis by regulating the activation of HSCs.

Innovations and breakthroughs

This is the first study to characterize the role of Notch signaling in liver fibrosis. This finding indicated that Notch3 may contribute to liver fibrosis by regulating the activation of HSCs.

Applications

This study showed that the selective interruption of Notch3 by siRNA decreased the expression of myofibroblastic marker in hepatic stellate cell-T6 cells, which provided a potential novel therapeutic target for liver fibrosis.

Peer review

This is a very interesting study aimed at investigating the role of the Notch signaling pathway in liver fibrosis. The text is generally well written, with structured abstract and organized sections. The manuscript has scientific value since it includes original information about a relevant topic.

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BRIEF ARTICLE

Endoscopic stenting and concurrent chemoradiotherapy for advanced esophageal cancer: A case-control study

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Abstract

AIM: To evaluate the role of endoscopic stenting with or without concurrent 3-dimensional conformal chemoradiotherapy (3D-CRT) in patients with inoperable esophageal cancer.

METHODS: Advanced esophageal cancer patients indicated for esophagectomy received esophageal stents. A part of patients completed 3D-CRT after stenting. Efficacy was assessed by endoscopy and computed tomographic scan before and 4 wk after completion of the treatment. The median survival, 3D-CRT toxicity and complications were compared between 3D-CRT and control groups.

RESULTS: From 1999 to 2008, 99 consecutive patients with T3/T4 disease and unsuitable for esophagectomy were placed with esophageal stents. Sixty-seven patients received 3D-CRT, while 36 patients treated with endoscopic stents alone were recruited as controls. After 3D-CRT treatment, the median tumor volume of 3D-CRT patients were reduced significantly from 43.7 ± 10.2 cm^3 to 28.8 ± 8.5 cm^3 ($P < 0.05$). The complete and partial response rate was 85.1%, and no response was 14.9%. After 3D-CRT, the incidence rate of T2 and T3 disease evident on CT scan increased to 78.4% while T4 decreased from 66.7% to 21.6% ($P < 0.05$). 3D-CRT Karnofsky Performance Status improved in 3D-CRT patients compared with the control group ($P = 0.031$). 3D-CRT patients had a longer survival than the control group (251.7 d *vs* 91.1 d, $P < 0.05$). And the median half-year survival rate in 3D-CRT group (91%) was higher than in the control group (50%, $P < 0.05$). The most common toxicity was leukocytopenia in the 3D-CRT group (46.7% *vs* 18.8%, $P = 0.008$). The control group had a higher rate of restenosis than the 3D-CRT group (81.3% *vs* 9.0%, $P < 0.05$). The rate of nephrotoxicity was increased in 3D-CRT as compared with the control group (31.3% *vs* 15.6%, $P < 0.05$).

CONCLUSION: 3D-CRT can improve dysphagia in patients with inoperable esophageal carcinoma. 3D-CRT combined with stenting results in better survival as compared with endoscopic stents used alone.

Key words: Esophageal Cancer; Stents; Chemoradiotherapy; Three-dimensional imaging; Case control study

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INTRODUCTION

Esophageal cancer is one of the most common malignant tumors with a high mortality rate in almost a half of the cases, and is the fourth leading cause of cancer-related deaths in China[1,2]. Most patients have been already in an advanced stage at the diagnosis of this aggressive malignancy. At least 60% of patients are unsuitable for surgical resection either due to the advanced stage or their comorbidity[3]. At this stage, esophageal carcinoma has infiltrated surrounding tissues and caused esophageal stenosis, and even esophagotracheal fistula in some cases. These patients could only be treated with palliative procedures which play an important role in improving the patient's life quality. The current palliative means include radiotherapy, chemotherapy and esophageal stent placement. However, esophagitis is usually unavoidable after radiotherapy, and once esophagitis occurred, dysphagia would be exacerbated[4]. Some patients even can not swallow liquid diet, some can not endure the chemotherapy and radiotherapy for their poor nutritional status. Stents placement has become a safe and effective palliation for dysphagia due to malignant esophageal obstruction or strictures after radiotherapy[5]. A randomized trial demonstrated that the combination of endoscopic stenting with additional radiation and chemotherapy could improve the survival of advanced esophageal cancer patients[6,7]. We evaluated the feasibility and efficacy of esophageal stenting combined with simultaneous radiotherapy and chemotherapy in the treatment of advanced esophageal cancer.

MATERIALS AND METHODS

Patients

All esophageal cancer patients treated in Qingdao Municipal Hospital from January 1999 to September 2008 were identified. The care of esophageal cancer patients was planned under the auspices of a multidisciplinary gastrointestinal disease management team which includes surgical oncologists, medical oncologists, gastroenterologists, pathologists, interventional radiologists, and radiation oncologists. This study was approved by the Ethics Committee of our institution and informed consent was obtained from all the patients before enrollment.

Clinical staging was performed with endoscopy and endoscopic ultrasound and computed tomography (CT). The tumor, node, metastasis and staging classification used for this analysis were defined according to the American Joint Committee on Cancer staging system version 6.0[8].

Inclusion criteria for the study were: (1) esophageal cancer at stage Ⅲ or Ⅳ unsuitable for esophagectomy; (2) symptoms of dysphagia ≥ grade 3 (Table 1); (3) tumors were mainly located in the esophagus; (4) adequate bone marrow (white blood cell > 3.5×10^9/L, hemoglobin > 90 g/L, platelet count > 100×10^9/L), and hepatic (bilirubin 1.5 times the upper limit of the normal value) and renal functions (calculated creatinine clearance > 50 mL/min or creatinine < 2 mg/dL) were examined before administration of chemotherapy; (5) a minimum life expectancy of 5 mo; and (6) stay in hospital during the entire chemoradiotherapy treatment course.

Table 1 Dysphagia assessment

Grade
Asymptomatic
Difficulty in swallowing solid food but able to swallow semisolid food
Difficulty in swallowing solid food, but able to swallow liquid one
Difficulty in swallowing liquid
Inability to swallow anything, including saliva

Patients were excluded because: (1) with tumors predominantly located in the stomach; (2) with prior treatment, including surgery, chemotherapy, or radiotherapy; and (3) with tumors infiltrating the tracheobronchial tree found on CT.

Data collected included patient clinical demographics, Karnofsky Performance Status, dysphagia grade, 76% meglumine diatrizoate compound swallow esophagogram (esophageal strictures) findings, endoscopic findings (tumor length at initial endoscopy, primary tumor, and lymphadenopathy location), tumor histology, and results of CT scan of the chest and abdomen with intravenous contrast.

Procedure of stenting

The procedure was performed under local anesthetic spray, with intravenous sedation when required. Endoscopy was done to determine precisely the site and length of stenosis. All patients with strictures underwent dilatation with 12-14 mm flexible rubber Savary-Guillard dilators before stent placement. Once this stricture was successfully dilated, a distal hemoclip (resolution clip, Boston Scientific, United States) was placed 2 cm below the area of stricture. Then endoscope was advanced farther and a flexible guidewire was placed into the second portion of the duodenum. The covered self-expanding Titanium Nickel alloy mesh stent (MTN, Nanjing Microinvasive Medical Inco., Nanjing, China) has a polyester at its midsection and its proximal end is flared to a diameter of 25 mm. The stent was loaded onto a dedicated applicator (12-14 mm depending on the diameter of stent) with an atraumatic dilator tip and was placed under continuous fluoroscopic guidance using the distal hemoclip as a mark. The length chosen was at least 2 cm longer than the stenosis. An 18 mm-diameter stent was used for severe strictures; and a 21 mm-diameter stent was used for moderate strictures.

Three-dimensional conformal chemoradiotherapy

Three-dimensional conformal chemoradiotherapy (3D-CRT) protocol was used. CT scans displayed isodose distributions and directly obtain dose-volume histograms. The primary tumors as well as the loco-regional lymph nodes were irradiated with an International Commission on Radiation Units and Measurements reference dose of 45.0 Gy in 25 fractions with 1.8 Gy/d using a LINAC

Table 2 Clinical and demographic characteristics of 3-dimensional conformal chemoradiotherapy-treated patients and controls

	3D-CRT (n = 67)	Control (n = 32)	P value
Gender			
Male	53	24	0.796
Female	14	8	
Age (mean ± SD, yr)	56.3 ± 12.7	58.6 ± 12.1	0.435
Karnofsky performance status			
50-70	37	21	0.386
10-49	30	11	
Dysphagia grade			
3	6	4	0.673
4	46	23	
5	15	5	
Stage at diagnosis			
Ⅲ	56	29	0.539
Ⅳ	11	3	
Tumor type			
SCC	53	23	0.579
Adenocarcinoma	6	4	
Unspecified	8	6	
Location			
Thoracic - middle	42	20	1.000
Thoracic - lower	25	12	
Gross tumor volume (mean ± SD, m^3)	44.6 ± 10.2	48.5 ± 11.1	0.077
Length (mean ± SD, cm)	7.3 ± 1.6	7.1 ± 1.8	0.299

SCC: Squamous cell carcinoma; 3D-CRT: 3-dimensional conformal chemoradiotherapy.

6-MV X-ray unit (Varian, CA, United States), and the total dose was adjusted according to patients' tolerance. Chemotherapy regimens consisted of cisplatin 60 mg/m^2 infusion on day 1 and day 22, plus continuous infusion of 5-fluorouracil at 200 mg/m^2 per day from day 1 to day 42.

Clinical response criteria

The clinical response to treatment was categorized as a clinically complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). CR was defined as the disappearance of all clinically detectable lesions; CR of primary tumors was defined as the disappearance of all visible lesions, including ulceration, with no microscopic evidence of tumor in randomly obtained biopsy specimens from the previous lesion sites. PR was defined as either a reduction exceeding 50% of the initial sizes (products of dimensions) of all measurable tumors according to CT or esophagraphy. A new lesion or more than a 25% increase of the original tumor size was defined as PD. All other conditions were categorized as SD. Chest CT was repeated every 4 wk to assess the response of the tumor. Endoscopy or abdominal CT was repeated if necessary.

Statistical analysis

The data was analyzed using SPSS 13.0, mean ± SD were calculated. Continuous variables were analyzed using Student's t test. Categorical variables were compared using χ^2 test or Fisher's exact test, whichever applicable. Repeated measure analysis (two-way ANOVA) was also used. Survival curves were estimated by the Kaplan-Meier method. A P value of 0.05 or less was considered significant.

RESULTS

Patient characteristics

Among the 146 patients who were diagnosed as having inoperable esophageal cancer, 111 (76.0%) were eligible for treatment with palliative 3D-CRT. Nine patients did not complete 3D-CRT because of disease progression. One patient died of severe bleeding 12 d after 3D-CRT. Two patients developed febrile neutropenia, and stopped the chemotherapy. Thirty-two patients did not undergo 3D-CRT after stents placement because their preference, these patients were regarded as the control group. Sixty-seven patients completed the 3D-CRT segment with a total radiation dose over 40Gy. Among these patients, 38 received 40Gy, and 29 received 50-55Gy. These patients were enrolled as the 3D-CRT treatment group. The clinical and demographic characteristics of the patients in 3D-CRT and control groups are shown in Table 2. There was no obvious difference in the dysphagia grade, tumor type, tumor stage and tumor size between the two groups.

Response after 3-dimensional conformal chemoradiotherapy

After 3D-CRT treatment, the median tumor volume of the 67 patients measured by CT scan reduced significantly from 43.7 ± 10.2 cm^3 to 28.8 ± 8.5 cm^3 ($P < 0.05$). The complete or partial response (> 50% reduction of tumor volume) was observed in 57 patients (85.1%), and no response in 10 patients (14.9%). The cases of T2 and T3 disease evident on CT scan had increased from 0 to 40 (78.4%) after 3D-CRT, and that of T4 decreased from 34 (66.7%) to 11(21.6%) ($P < 0.05$). After 3D-CRT, the number of cases of dysphagia at grade ⩽ 3 among the 67 patients increased significantly from 6 (9.0%) to 55 (82.1%), while the number of dysphagia at grade ⩾ 4 reduced from 61(91.0%) to 12(17.9%) ($P < 0.05$). Karnofsky Performance Status improved in the 3D-CRT patients compared with the control group ($P = 0.031$). The result of response assessed by CT scan and endoscopy is listed in Table 3.

Survival comparison

Patients treated with 3D-CRT seemed to have a better prognosis, with a longer survival duration (251.7 d *vs* 91.1 d, $P < 0.05$; Figure 1). The median six-month survival rate in 3D-CRT group (91%) was higher than in the control group (50%, $P < 0.05$, Table 4). The overall 1-year survival rate after 3D-CRT was 25%; none of the control patients survived more than 1 year. Although the mean number of hospital admissions was significantly higher in patients who received 3D-CRT (146 ± 48 d *vs* 87 ± 29 d, $P < 0.05$), the overall survival of the 3D-CRT group without hospitalization was statistically significantly higher than that of the control group (186 ± 33 d *vs* 59 ± 18 d, $P < 0.05$).

Table 3 Radiological and clinical evaluation after 3-dimensional conformal chemoradiotherapy

	Before 3D-CRT	After 3D-CRT	*P* value
Tumor staging			
T2 and T3	0	40 (78.4%)	< 0.05
T4	34 (66.7%)	11 (21.6%)	
Tumor volume (mean ± SD, cm^3)	43.7 ± 10.2	28.8 ± 8.5	< 0.05
Assessment of tumor response			
Complete and partial response		57 (85.1%)	
No response		10 (14.9%)	
Dysphagia grade			
≤ 3	6 (9.0%)	55 (82.1%)	< 0.05
≥ 4	61 (91.0%)	12 (17.9%)	
Karnofsky performance status			
50-70	37	49	0.031
10-49	30	18	

3D-CRT: 3-dimensional conformal chemoradiotherapy.

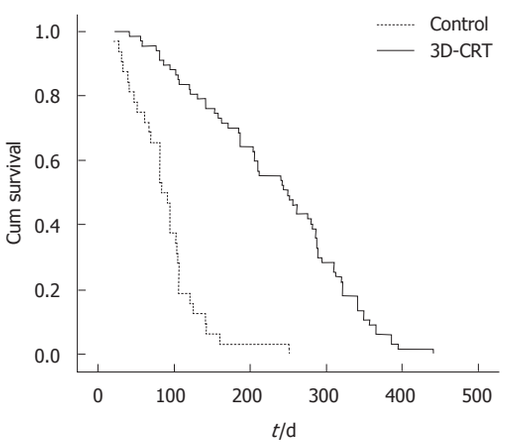

Figure 1 Comparison of overall survival between 3D-CRT and control groups. 3D-CRT: 3-dimensional conformal chemoradiotherapy.

Toxicity and complications

The toxicity profile is presented in Table 5. After 3D-CRT, the most common toxicity was leukocytopenia as compared with the control group without 3D-CRT treatment (46.7% *vs* 18.8%, $P = 0.008$). However, in the control group, the most common non-hematologic toxicities included a higher rate of restenosis compared with 3D-CRT group (81.3% *vs* 9.0%, $P < 0.05$). The incidence of nephrotoxicity in 3D-CRT was higher than in control group (31.3% *vs* 15.6%, $P < 0.05$).

DISCUSSION

About more than 90% of esophageal cancer patients are not clinically identified until at an advanced stage, their prognosis is the poorest among the patients with digestive carcinomas, with a 5-year survival rate below 10%[1]. Because of malnourishment due to comorbid conditions in advanced carcinoma, more than 50% of esophageal cancer cases are unresectable[9]. A case-control study showed that the median survival time for patients with advanced esophageal cancer was only 3-5 mo[10]. However, once these inoperable patients' physical condition was permitted, effective therapies should be performed. No matter which therapy is used, the primary purpose is to prolong the patients' survival time and improve their quality of life. In this study, we relieved the dysphagia with minimal morbidity and mortality, and then choose the suitable management to prolong the patients' life span and improve their quality of life.

Table 4 Comparison of clinical and survival outcomes between 3-dimensional conformal and control groups

Outcome	3D-CRT (*n* = 67)	Control (*n* = 32)
Median survival (d)	251.7	91.1
Overall survival at a half year	91%	50%
Over survival at 1 year	25%	0
Total hospital stay (mean ± SD, d)	146 ± 48	87 ± 29
Median hospitalization-free survival (mean ± SD, d)	186 ± 33	59 ± 18

3D-CRT: 3-dimensional conformal chemoradiotherapy.

Table 5 Toxicity and complications in patients after 4-wk 3-dimensional conformal and control group after treatment *n* (%)

	3D-CRT (*n* =67)	Control (*n* = 32)	*P* value
Hematologic			
Anemia	33 (49.3)	11 (34.4)	0.198
Thrombocytopenia	21 (31.3)	7 (21.9)	0.475
Leukocytopenia	31 (46.7)	6 (18.8)	0.008
Non-hemotologic			
Reflux esophagitis	28 (41.8)	15 (46.9)	0.669
Pectoralgia	29 (43.3)	14 (43.8)	1.000
Perforation	16 (23.9)	12 (37.5)	0.232
Restenosis	6 (9.0)	26 (81.3)	< 0.05
Nephrotoxicity	21 (31.3)	5 (15.6)	< 0.05
Shift and brush off of stent	8 (11.9)	5 (15.6)	0.751

3D-CRT: 3-dimensional conformal chemoradiotherapy.

Recently, 3D-CRT appeared to be a promising treatment even for esophageal carcinoma patients with distant metastasis[11]. Because CDDP and 5-FU have synergistic effects and also act as radiosensitizers, these agents were considered to be particularly effective in combination with radiotherapy[12].

In our study, we focused on the 3D-CRT effectiveness in the inoperable esophageal cancer patients. Ten years ago, we could only place stents to palliate dysphagia among these patients. And in recent years after stents placement, 3D-CRT could be performed as long as patient's physical condition allowed, and whenever their dysphagia was relieved, the malnutrition of the patients was ameliorated. For these inoperable patients, esophageal stents placement not only improved their quality of life, but made the 3D-CRT completion possible as well. In our study, compared with the patients treated with stenting only, at least 25% of the inoperable patients after 3D-CRT survived 1-year, and their six-month survival rate was also higher. 3D-CRT could reduce the tumor volume

and improve Karnofsky Performance Status in the inoperable patients. Combined with 3D-CRT, it seemed that the esophageal stent placement is the adjuvant therapy with CRT. We found that stenting combined with 3D-CRT could improve the inoperable patients' quality of life, and prolong their survival. Fietkau[4] thought that simultaneous CRT should be considered as the standard treatment for inoperable carcinoma of the esophagus with the median survival time between 13 and 18 mo. The reason why Fietkau's result[4] was better than ours may be that there were more inoperable stage Ⅲ/Ⅳ patients in our study as compared with the stage Ⅱ/Ⅲ patients in Fietkau's study.

3D provides the superior dose distributions in the target volume with markedly reduced morbidity to the surrounding normal tissues. Minsky found that increasing the percutaneous radiation dose from 50.4 to 64.8 Gy did not result in better survival[13]. Part of the patients in our study received radiation dose of less 40Gy due to the complications such as perforation or esophagitis. Although there is still no consensus of the optimal dose of radiation within the framework of simultaneous RCT, there has been a common opinion that the higher dose of radiation the more therapy-related deaths. In our study, our therapeutic purposes were not to remit or downstage the tumor, but to alleviate the pain and improve the quality of life of the patients. Moreover, 3D-CRT with covered metal stenting would cause a relatively higher rate of late complications such as stent migration, hemorrhage, and gastroesophageal mucosal prolapse. The results from some studies about the safety of self-expandable metallic stents for patients who have undergone chemoradiotherapy were controversial[14-16]. Therefore, more studies should be conducted.

In our study, there was no difference of complications such as perforation, shift and brush off of stent and pectoralgia between the 3D-CRT group and the control group with only stenting. Because we did not determine whether lymph node metastasis occurred in these patients without biopsies from esophagectomy, we did not compare the patients with and without lymph node metastasis. Those who could not be treated by 3D-CRT due to poor physical conditions served as controls, and there might be selection bias in this study.

Fietkau[4] pointed out that CRT could increase the treatment-related toxicity, in particular, the hematological side effects. This result was in agreement with ours, which was caused by 3D-CRT such as leukocytopenia and nephrotoxicity. After 3D-CRT, more leukocytopenia and nephrotoxicity occurred, but less restenosis was found as compared with the control group. Therefore, among the patients with leukocytopenia and liver and renal dysfunctions, caution should be exercised in the application of 3D-CRT.

Furthermore, more patients were relieved from dysphagia during the later stages of life after 3D-CRT treatment. 3D-CRT is beneficial for the majority of patients with advanced esophageal cancer in the improvement of quality of life and survival. In our study, endoscopic stenting with 3D-CRT for advanced esophageal cancer patients could relieve the dysphagia and prolong the survival, and decrease the incidence of restenosis as well. Therefore, more efficient and combined management should be explored and studied to improve the survival and quality of life of the patients with advanced esophageal cancer.

COMMENTS

Background

Placement of esophageal stents in patients with advanced esophageal cancer can improve the symptoms of dysphagia. However, the safety of esophageal stents for patients receiving chemoradiotherapy is controversial. The authors evaluated the morbidity and mortality after self-expandable metallic stent placement with and without 3-dimensional conformal chemoradiotherapy (3D-CRT) in advanced esophageal cancer patients unsuitable for surgery.

Research frontiers

In this study, once the dysphagia in advanced esophageal cancer patients was alleviated, concurrent 3D-CRT was carried out. 3D-CRT combined with esophageal stenting could improve the symptoms of dysphagia in patients with inoperable esophageal carcinoma and could prolong their survival as well.

Innovations and breakthroughs

This study showed that self-expandable metallic stent placement with concurrent 3D-CRT could improve the symptoms of dysphagia and result in better survival as compared with endoscopic stenting used alone in the patients with inoperable esophageal carcinoma.

Applications

The combined treatment of endoscopic stenting and 3D-CR could be applied in patients with inoperable advanced esophageal cancer.

Terminology

Three-dimensional conformal chemoradiotherapy (3D-CRT) is a mode of high precision radiotherapy. It is a complex process that begins with the creation of individualized, 3D digital data sets of patient tumors and normal adjacent anatomy. These data sets are then used to generate 3D computer images and to develop complex plans to deliver highly "conformed" (focused) radiation while sparing normal adjacent tissues. The radiation beam could focus on a higher radiation dose to the tumor while minimizing radiation exposure to healthy cells.

Peer review

In this study, the authors examined the efficacy and toxicity/complication of esophageal stenting thereafter 3D-CRT for patients with inoperable esophageal cancer. The results of this study revealed that esophageal stenting after 3D-CRT induced no severe complication, prolonged their survival and effectively improved their symptoms of dysphagia than stenting alone in patients with inoperable advanced esophageal cancer.

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CASE REPORT

A rare case of langerhans cell histiocytosis of the gastrointestinal tract

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Abstract

Langerhans cell histiocytosis (LCH) is a group of idiopathic disorders characterized by the proliferation of specialized, bone marrow-derived langerhans cells and mature eosinophils. The clinical spectrum ranges from an acute, fulminant, disseminated disease called Letterer-Siwe disease to solitary or few, indolent and chronic lesions of the bone or other organs called eosinophilic granuloma. Involvement of the gastrointestinal tract is very rare in LCH. We present the case of a 53-year-old woman referred by her primary care physician for a screening colonoscopy. A single sessile polyp, measuring 4 mm in size, was found in the rectum. Histopathological examination revealed that the lesion was relatively well circumscribed and comprised mainly a mixture of polygonal cells with moderate-to-abundant pink slightly granular cytoplasm. The nuclei within these cells had frequent grooves and were occasionally folded. Immunohistochemical staining was positive for CD-1a which confirmed the diagnosis of LCH. On further workup, there was no evidence of involvement of any other organ. On follow up colonoscopy one year later, there was no evidence of disease recurrence. Review of the published literature revealed that LCH presenting as solitary colonic polyp is rare. However, with the increasing rates of screening colonoscopy, more colonic polyps may be identified as LCH on histopathology. This underscores the importance of recognizing this rare condition and ensuring proper follow-up to rule out systemic disease.

Key words: Langerhans cells; Histiocytosis; Colonic polyp; CD-1a; Eosinophilic granuloma; Screening; Colonoscopy

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INTRODUCTION

Langerhans cell histiocytosis (LCH) is a group of idiopathic disorders characterized by the proliferation of specialized, bone marrow-derived langerhans cells (LCs) and mature eosinophils. The clinical spectrum of LCH ranges from an acute, fulminant, disseminated disease (called Letterer-Siwe disease) to solitary or few indolent and chronic lesions of the bone or other organs called (eosinophilic granuloma). The intermediate clinical form, called Hand-Schüller-Christian disease, is characterized by multifocal, chronic involvement and classically presents as a triad of diabetes insipidus, proptosis and lytic bone lesions.

LCH is a rare disease of the pediatric population,

with an estimated annual incidence of 4-5 per million. More than two-thirds of cases have single system disease with bones and skin as the most commonly involved sites[1,2]. Other organs involved are the lung, liver, spleen, bone marrow, lymph nodes and the hypothalamic-pituitary region[3]. Involvement of the gastrointestinal (GI) tract is very rare in LCH, especially in adults, with only a few isolated case reports available in English-language literature[4].

The pathogenesis of LCH is unknown. An ongoing debate exists over whether this is a reactive or neoplastic process. From the histopathological viewpoint, the demonstration of LC (Birbeck) granules by electron microscopy remains the "gold standard" for diagnosis of the phenotype, but expression of the CD1a antigen on lesional cells also provides the basis for a definitive diagnosis[5].

CASE REPORT

We present a case of a 53-year-old woman with a history of hyperlipidemia who was referred by her primary care physician for a screening colonoscopy. She was essentially asymptomatic and did not report any abdominal pain, blood in stool, change in bowel habits, dysphagia, nausea, or vomiting. Findings of physical examination were unremarkable.

On colonoscopy, quality of bowel preparation was excellent. A single sessile polyp measuring 4 mm in size was found in the rectum (Figure 1). It was removed by cold snare polypectomy. The procedure was performed without complications.

Histopathological examination of the polyp revealed that the lesion was relatively well-circumscribed and comprised mainly a mixture of polygonal cells. These cells had moderate-to-abundant pink slightly granular cytoplasm. The lesion also contained inflammatory cells. The inflammatory populations included eosinophils, lymphocytes and neutrophils. The nuclei within the larger cells had frequent grooves and were occasionally folded, suggesting the presence of LCs (Figure 2). Immunohistochemical staining showed histiocytes with cytoplasmic and membranous staining for CD-1a (Figure 3). Histiocyte cytoplasm was positive for S-100 and negative for prekeratin on immunohistochemistry. Electron microscopy was not performed to document presence of Birbeck granules as staining for CD-1a was strongly positive and provided confirmation for the diagnosis[5].

The patient was referred for medical oncology evaluation for this unusual pathologic finding with malignant potential. A computerized tomography (CT) scan of the chest, abdomen, and pelvis was performed to rule out any metastasis through staging evaluation. The CT scan showed no evidence of metastasis, although there were fibroids in the uterus and few cystic masses in the ovaries. Some prominent lymph nodes, which appeared to be benign, were observed in the right and left axillae and sub carina. Further evaluation by an oncologist and a gynecologist revealed that these lesions were benign.

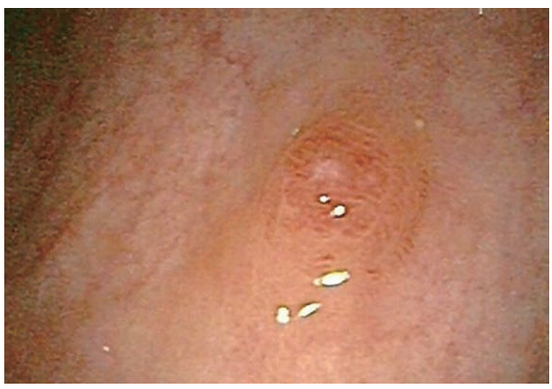

Figure 1 Endoscopic view of the rectal polyp.

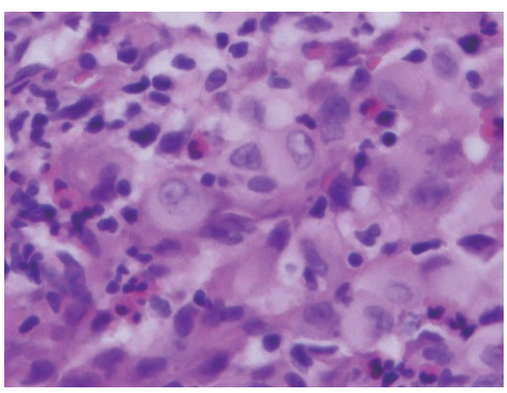

Figure 2 Rectal biopsy with histiocytic infiltrate in the lamina propria. Note eosinophils (× 400).

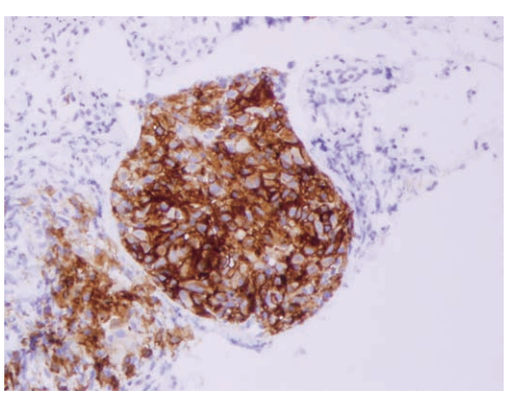

Figure 3 CD- I **a immunostain.** Histiocytes show positive cytoplasmic and membranous staining (× 200).

Therefore, we found no evidence of disseminated LCH.

The patient remained asymptomatic, and a follow-up colonoscopy was performed 1 year later. A single sessile polyp, measuring 4 mm, was found in the rectum. Another single sessile polyp, measuring 5 mm, was found in the proximal ascending colon. Histopathological examination showed that the polyps were hyperplastic and tubular adenomatous polyps, respectively.

DISCUSSION

In the GI tract, LCH involvement of the stomach[6-8],

small intestine[9-11], colon[4,12,13] and perianal skin[14,15] has been reported. Rectal LCH, proven by histopathology, has been described in infants presenting with bloody diarrhea. In most cases, rectal LCH in infants is indicative of widespread, multisystem disease[16].

Among the 6 reported cases of LCH of stomach, all patients were in the fourth and fifth decade of life, and the male-to-female ratio was 1:1; five patients presented with abdominal pain, and one was asymptomatic. The gastric lesion was described as a large, flat raised area in 1 case and a single polyp in 1 case. In other 2 cases, the gastric lesion was described as an ulcerating mass and multiple polyposis, respectively. Histopathological examination of lesions from 2 cases revealed malignant features[8,17]. In the case reported by Terracciano *et al*[8], the patient presented with abdominal pain and weight loss; the tumor was located on the lesser curvature of the stomach. During laparotomy, the tumor was found to have invaded the distal pancreas. Some areas of the tumor had high mitotic indexes and cytologic atypia. Most neoplastic cells were intensely positive for vimentin, S-100 and CD 1a on immunohistochemistry. Two months after surgical resection, the patient died at home. The cause of death was unclear. The remainder of the cases did not show histopathological or clinical malignant features.

Small bowel LCH is rare, with only 3 cases being reported in infants[10,18] and 1 in an adult[11]. All infants with small bowel LCH presented with diarrhea, weight loss, and failure to thrive. Laboratory findings were significant for anemia and hypoalbuminemia, and the duodenum was always involved. All patients simultaneously or subsequently developed widespread, multisystem involvement, requiring chemotherapy. Two patients responded well to chemotherapy with complete remission, but 1 succumbed to the disease after poor response to treatment and finally refusing chemotherapy[18]. In adults, only 1 proven case of small bowel LCH has been reported. LCH occurred in a patient who had undergone right hemicolectomy for steroid-refractory Crohn's disease; this patient presented with worsening diarrhea, abdominal pain, and weight loss. Barium follow-through showed extensive mucosal infiltration of the small bowel wall, and the duodenal biopsy result was consistent with a diagnosis of LCH. Results of bone marrow examination were consistent with chronic myelomonocytic leukemia. The authors concluded that in this case, LCH was a complication of Crohn's disease because of the increased incidence of myeloproliferative disorders in inflammatory bowel disease[11,19].

In children, colonic involvement in LCH is extremely rare. Only 1 case of LCH has been reported in an infant who died of widespread multisystem disease consistent with Litterer-Siwe disease; LCH was diagnosed post-mortem in this case[13]. In adults, there have been only 2 reported cases of colonic LCH presenting as isolated polyps in English-language literature[4,12]. Both patients were essentially asymptomatic, and polyps were detected during screening colonoscopy. In both cases, diagnosis was confirmed by positive immunochemical staining for CD1a antigen. Extensive workup in both cases did not reveal involvement of any other organ. In the case presented herein, a polyp was located in the rectum, and diagnosis was supported by the presence of an eosinophilic infiltrate and LCs. The diagnosis was confirmed by strong immunochemical staining for CD1a antigen. The workup to rule out involvement of other organs was negative, and repeat colon examination did not reveal recurrence.

In conclusion, LCH of the GI tract is extremely rare. Colonic involvement in adults usually presents as a solitary polyp without multisystem disease. With the increasing rates of screening colonoscopy, more colonic polyps may be identified as LCH on histopathology. This underscores the importance of recognizing this rare condition and ensuring proper follow-up to rule out systemic disease.

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CASE REPORT

Pseudomelanosis duodeni associated with chronic renal failure

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Abstract

Pseudomelanosis duodeni (PD) is a rare dark speckled appearance of the duodenum associated with gastrointestinal bleeding, hypertension, chronic heart failure, chronic renal failure and consumption of different drugs. We report four cases of PD associated with chronic renal failure admitted to the gastroenterology outpatient unit due to epigastric pain, nausea, melena and progressive reduction of hemoglobin index. Gastroduodenal endoscopy revealed erosions in the esophagus and stomach, with no active bleeding at the moment. In addition, the duodenal mucosa presented marked signs of melanosis; later confirmed by histopathological study. Even though PD is usually regarded as a benign condition, its pathogenesis and clinical significance is yet to be defined.

Key words: Pseudomelanosis duodeni; Chronic renal failure; Diabetes mellitus; Anti hypertensive drugs; Gastroduodenal endoscopy

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INTRODUCTION

Melanosis duodeni was first described in 1976 and refers to a rare endoscopic appearance of discrete speckled black pigmentation of duodenal mucosa, which was initially postulated to represent a form of stored iron[1]. The term melanosis gives a false idea that the pigment is produced by melanocytes, justifying renaming this endoscopic finding as pseudomelanosis duodeni (PD)[2]. Nevertheless, the clinical significance of this condition is yet to be established. PD is more common in the sixth and seventh decade of life with a female predominance. It has been postulated to be associated with gastric hemorrhage, certain chronic illnesses, such as diabetes mellitus, hypertension, as well as various medications, such as sulfur-containing antihypertensive agents and ferrous sulfate[3-5].

CASE REPORT

Case 1

A 66-year-old woman was admitted with a history of melena and a progressive decrease of hemoglobin index. She presented with a history of diabetes mellitus and

systemic arterial hypertension for > 30 years, with a diagnosis of chronic renal failure in the last 6 mo, treated with hemodialysis. She was on long-term treatment with angiotensin-converting enzyme inhibitors, furosemide, ferrous sulfate, folic acid and insulin. Gastroduodenal endoscopy was performed and revealed multiple superficial erosions in the stomach without signs of recent bleeding. In addition, in the duodenal mucosa, pigmented lesions with a speckled pattern were evident (Figure 1). Biopsies revealed numerous macrophages containing brown pigment granules in their cytoplasm within the lamina propria (Figure 2). Staining of the specimen with Masson Fontana stain demonstrated iron-containing deposits inside the macrophages (Figure 3).

Case 2

A 37-year-old woman with a history of renal transplantation (hypertensive nephropathy) had been previously treated with furosemide, propranolol, hydralazine and ferrous sulfate. She was referred to the gastroenterology section due to classical gastroesophageal reflux disease symptoms. She was submitted to gastroduodenal endoscopy that revealed reflux esophagitis and diffuse, multiple, dark brown spots in the duodenum. Biopsies were taken and light microscopy confirmed the presence of brown pigment granules in the lamina propria.

Case 3

A 70-year-old woman with diabetes and long-term systemic arterial hypertension developed chronic renal failure that was initially treated conservatively with calcium channel blockers, propranolol, α-methyldopa, furosemide and glibenclamide. She underwent a left nephrectomy due to nephrolithiasis. Progressive reduction of hemoglobin index was observed during follow-up, therefore, gastroduodenal endoscopy was performed, revealing multiple non-actively bleeding superficial gastric erosions and pigmented lesions in the duodenum. Biopsies were collected and histopathological examination showed features of PD.

Case 4

A 30-year-old woman with diabetes started complaining of epigastric pain, nausea and vomiting 1 mo after renal and pancreatic transplantation (diabetic nephropathy). She had been previously taking insulin, and after the transplant, she started tracolimus, mycophenolate and corticosteroid therapy. Gastroduodenal endoscopy revealed cytomegalovirus (CMV) esophagitis and multiple small pigmented duodenal spots. Biopsies were taken and histopathological examination confirmed the diagnosis of PD. Another gastroduodenal endoscopy was performed after CMV treatment, and demonstrated total regression of the esophageal lesions but no changes in the aspect of the duodenal mucosa.

DISCUSSION

PD represents a fine granular brown material inside the macrophage lysosomes in the lamina propria around the tips of the duodenal villi, detected by histochemical staining and electron microscopy. It has been postulated that this heterogeneous pigment may represent a deposit of melanin-like substances, hemosiderin, lipomelanin and lipofuscin[6,7]. Even though iron (ferrous sulfide) is the main pigment compound, varying amounts of sulfur, calcium, potassium, aluminum, magnesium and silver can also be detected[5,8]. The color of the pigment could represent various degrees of auto-oxidation of ferrous sulfide[2,6].

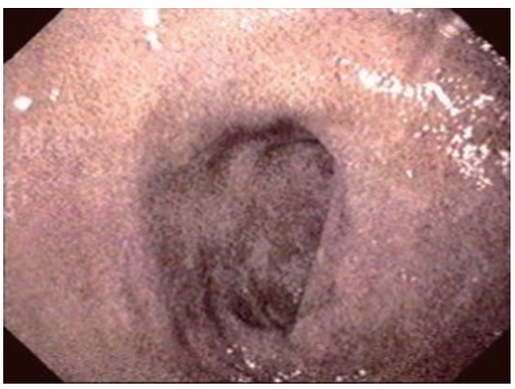

Figure 1 Endoscopic picture showing multiple dark brown spots in the duodenum.

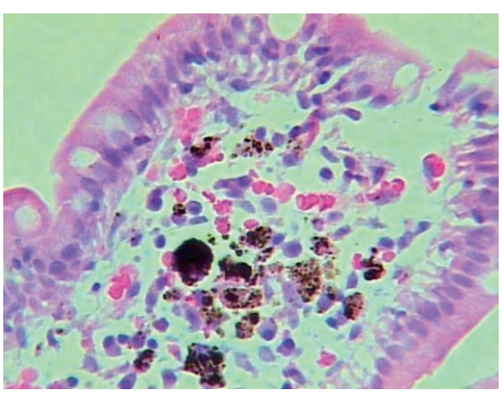

Figure 2 Many macrophages containing brown pigmented granules within the lamina propria (hematoxylin and eosin stain, x 200).

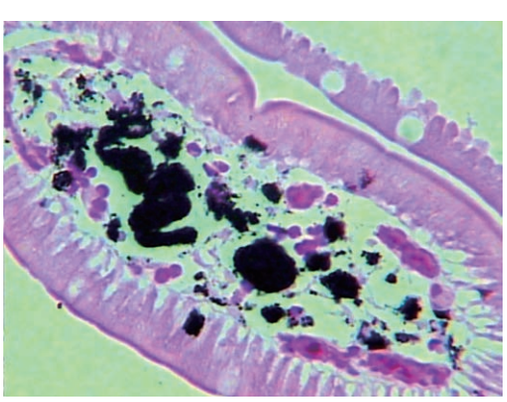

Figure 3 Iron deposits inside macrophage cytoplasm (Masson-Fontana stain, x 200).

The pathogenesis still remains unclear. It could be related to iron deposition secondary to intramucosal hemorrhage or impaired intramucosal iron transport after oral ferrous sulfate supplementation[6,9]. Iron sulfide storage can also be a product of an acquired inherent defect in macrophage metabolism. In that regard, the pigment present in the duodenal mucosa has also been shown to be partially associated with impaired macrophage metabolism of drugs containing cyclic compounds such as phenols, indoles and skatoles[8].

All four patients had undergone previous gastroduodenal endoscopy without any pathological findings, suggesting that this condition might be acquired rather than congenital, which is in keeping with previous reports[5]. Importantly, it must be stressed that this entity might be identified histologically even before it becomes endoscopically visible, making it difficult to establish a temporal association between disease onset and endoscopic manifestation[10].

In this report, all patients were female, with chronic renal failure, and taking antihypertensive drugs. Of note, only two patients were taking oral iron supplements. The biopsy specimens were positive for hematoxylin and eosin and Masson-Fontana stains but not reactive for Pearl' s stain, suggesting a melanin-like compound. Although Pearl's stain is a classic method for demonstrating iron in tissues, there is a possibility of a false-negative reaction if an iron oxide compound is present instead of iron sulfide[11,12].

In conclusion, these findings suggest that the duodenal involvement can occur in the absence of a history of oral iron supplementation. Importantly, although the long-term clinical impact of these depositions remains unclear, these endoscopic findings still do not require any specific treatment or recommended follow-up.

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January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
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London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
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Montreal, Canada

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International Conference on Nutrition and Growth 2012
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26th Annual New Treatments in Chronic Liver Disease
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Singapore, Singapore

April 15-17, 2012
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April 18-20, 2012
The International Liver Congress 2012
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April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

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Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
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May 19-22, 2012
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San Diego, CA 92121, United States

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June 18-21, 2012
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ic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

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

Issue with no volume

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

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

Books

Personal author(s)

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

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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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

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Conference paper

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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