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EDITORIAL

Estrogen, male dominance and esophageal adenocarcinoma: Is there a link?

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Abstract

Esophageal adenocarcinoma is a cancer with poor prognosis, and its incidence has risen sharply over recent decades. Obesity is a major risk factor for developing this cancer and there is a clear male gender bias in the incidence that cannot be fully explained by known risk factors. It is possible that a difference in the expression of estrogen, or its signaling axes, may contribute to this gender bias. We undertook a comprehensive literature search and analyzed the available data regarding estrogen and estrogen receptor expression, and the possible sex-specific links with esophageal adenocarcinoma development. Potentially relevant associations between visceral *vs* subcutaneous fat deposition and estrogen expression, and the effect of crosstalk between estrogen and leptin signaling were identified. We also found limited studies suggesting a role for estrogen receptor β expression in esophageal adenocarcinoma development. The current literature supports speculation on an etiological role for estrogen in the male gender bias in esophageal adenocarcinoma, but further studies are required.

Key words: Estrogen; Estrogen receptors; Male dominance; Esophageal adenocarcinoma

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INTRODUCTION

Esophageal carcinoma is the eighth most common cancer worldwide, and over the last three decades its incidence has risen significantly in all Western countries[1-4]. This change is entirely due to an increase in the adenocarcinoma subtype, and this has predominantly occurred in males[2,5-9]. Recent Western experiences report that the male:female ratio for patients undergoing esophagectomy for adenocarcinoma now exceeds 8:1. However, the identified risk factors for esophageal adenocarcinoma, including gastroesophageal reflux disease, Barrett's esophagus, obesity, alcohol, and tobacco consumption cannot adequately explain this profound gender difference. The dramatic gender difference for esophageal adenocarcinoma suggests there should be a gender-related mechanism underpinning this phenomenon. Estrogens, the primary female sex hormones, are mechanistically linked to aspects of cancer risk and cancer development. Therefore it seems reasonable to consider that estrogens might

contribute towards the gender difference for esophageal adenocarcinoma.

A link between estrogen-activated signaling and carcinogenesis in many organs, including mammary glands[10], ovaries and colon[11] has been clearly defined, although it is unclear whether a similar connection exists for the esophagus, and esophageal adenocarcinoma in particular. Additionally, estrogen is actively involved in the regulation of metabolism in adipose tissues[12], and it can be synthesized locally by activated aromatase in adipocytes in both men and women[13-15]. Involvement of estrogen signaling in regulation of adipose tissue metabolism indicates a possible connection between the effects of estrogen and male obesity - one of the main risk factors for esophageal adenocarcinoma. Given the established regulatory role for estrogen in carcinogenesis and metabolic homeostasis for other cancers, and the strong gender differences for the incidence of esophageal adenocarcinoma, it is plausible to suggest that the estrogen signaling network is involved in the progression of this cancer, and an understanding of estrogen and estrogen receptor (ER) roles in the regulation of carcinogenesis, and how this might be relevant in the esophagus, could provide a basis for developing either preventive measures or new treatments.

In this paper we review potential links between estrogen signaling and esophageal adenocarcinoma, to determine whether this might contribute to the dominance of esophageal adenocarcinoma in males. Literature pertinent to gender specific differences in estrogen synthesis, estrogen-regulated carcinogenesis, specific differences between ERs and signaling in cancer cells, and available information about estrogen signaling in esophageal adenocarcinoma is reviewed.

ESTROGEN IN WOMEN AND MEN: AGE RELATED CHANGES

In premenopausal women the ovaries are the principal source of estrogen[16]. Serum estradiol concentration is much higher in premenopausal women, compared to men, but decreases substantially after the menopause, and ultimately becomes lower than in elderly men[17]. Mass spectrometry has shown that average levels of serum estradiol in elderly men are approximately 73 pmol/L, whereas levels in postmenopausal women are markedly lower (about 15 pmol/L)[17].

When the ovaries cease to produce estrogens in postmenopausal women the main characteristics of estradiol function change, and it is produced in extragonadal sites, and acts locally at these sites as a paracrine or intracrine factor[16-20]. These sites are similar in men and postmenopausal women and include the mesenchymal cells of adipose tissue[13], osteoblasts and chondrocytes of bone[21], the vascular endothelium and aortic smooth muscle cells[22], and numerous sites in the brain[16,17,23].

Importantly, in men and postmenopausal women, circulating estrogens are not the main drivers of estrogen action, but locally produced estrogens originating in extragonadal sites are responsible for the majority of paracrine and intracrine effects of these hormones[20]. The total amount of estrogen synthesized by these extragonadal sites may be small, but the local tissue concentrations achieved are probably high and exert biological influence locally. This might impact on tumor biology. For example, it has been determined that the concentration of estradiol present in breast tumors in postmenopausal women is at least 20-fold higher than in the plasma. Aromatase inhibitor therapy is associated with a major decrease in intratumoral concentrations of estradiol and estrone and loss of intratumoral aromatase activity, which is followed by downregulation of cancer cell growth[10,24]. Local estrogen biosynthesis has also been demonstrated in men, where aromatase expression in adipose tissue is greatly increased by this process[25,26]. However, with respect to esophageal adenocarcinoma, no studies have evaluated whether the amount of estrogen synthesized in abdominal adipose tissue is sufficient to exert any paracrine effect in the esophagus.

ESTROGEN RECEPTORS: STRUCTURE AND FUNCTIONS

The effects of estrogens are mediated by their ligation to ERα and ERβ. ERα and ERβ both belong to the nuclear steroid/thyroid hormone receptor family and they are encoded by two distinct genes [encoding estrogen receptor 1 (*ESR1*) and *ESR2*] which are located on two different chromosomes 6q25.1 and 14q22-24[27,28]. ERα and ERβ have distinct cellular distributions and regulate separate sets of genes. ERα is predominantly expressed in female sex organs such as the breast, uterus and ovaries especially during the reproductive years. ERβ is widely expressed in many other tissues in both genders, but to a lesser degree in males compared to females[15,29]. Although the role of ERs in male physiology has long been neglected, there is growing evidence for estrogen involvement in multiple areas of male physiology[15-17].

The mechanism for ER signaling has been widely investigated. ERα and ERβ share common functional domains, with a conserved central DNA-binding domain which is often involved in receptor dimerization[30,31]. ERs possess two activation function domains; activation function-1 and activation function-2, with the former interacting with non-ER transcription factors, and the latter containing the ligand binding domain[31,32]. One of the most important differences between ERα and ERβ is that activation function-1 in ERβ lacks functional activity[30]. Also, it has been suggested that the main function of ERβ is to bind ERα and suppress its activation, so that ERα and ERβ as a dimer might exert inverse biological effects. Another difference between ERα and ERβ signaling is their interaction with the activator protein-1[32-35]. The activator protein-1 complex of Jun/Fos hetero- or homo-dimers is a key regulator of cell proliferation, with one of its target genes identified as cyclin

D1[33]. Depending on whether ERα or ERβ is activated, the activator protein-1 complex acts in a reciprocal fashion to stimulate or inhibit cell proliferation[35].

After binding estrogen, the receptor ligand-binding domain undergoes a conformational and surface-charge change that results in receptor dimerization. Ligand-binding is accompanied by the dissociation of intracellular ER from chaperone proteins, subsequently releasing the hormone/ER complex for attachment to estrogen response elements in the promoter region of target genes. The dimer then binds DNA to regulate gene expression at specific regions of the DNA named hormone response elements[31,35]. As a consequence, transcription of 17β-estradiol-responsive genes increases, and proliferation or differentiation of steroid-sensitive tissue is augmented. Although most steroid hormone receptors primarily localize to the nuclei, additional ERs have been identified in the cytoplasm and on the plasma membrane. Activation of cytoplasm signaling cascades has been detected after estrogen binding to its plasma membrane receptors[36].

Several isoforms of ERβ able to mediate estrogen signaling have also been found. The isoforms can exert diverse functions, and significantly complicate understanding of cellular responses to estrogens. ERβ isoforms can inhibit ERα transcriptional activity at the estrogen response elements and potentially reverse estrogen signaling[34]. A splice variant of ERβ, termed ERβcx, has been characterized[37]. ERβcx is expressed in the breast[38], the prostate and testis[37], the esophagus[39], and in gastric tissue[40]. Interestingly, ERβcx does not bind estrogen[41]. Instead it inhibits ERα from binding DNA, whilst it does not influence ERβ. The role and mechanism of ERβcx downstream signaling in esophageal tissue is largely unclear and needs to be further investigated.

ROLE OF ESTROGEN AND ESTROGEN RECEPTORS IN VARIOUS MALIGNANCIES: HARMFUL OR HELPFUL?

The biological significance of ERs in breast tumorigenesis has been studied extensively. In breast tumors, ER signaling promotes malignancy due to oncogenic mutations, sustained exposure of ERα with endogenous or exogenous estrogen, and abnormal coupling of estrogen-activated cytoplasmic machinery to growth and anti-apoptosis, all well established causative triggers of cancer in postmenopausal women[41]. Several large prospective studies have confirmed the role of estrogen in stimulation of breast tumor growth, and have demonstrated that the risk of breast cancer is increased in women taking estradiol after the menopause[42-44].

In females with breast cancer, ERα is instrumental in promoting cell proliferation and cancer progression, whereas ERβ exerts anti-proliferative effects by induction of cell cycle and growth arrest[34]. For instance, the downregulation of the cyclin D1 gene by ERβ prevents cellular progression from the G1 to S-phase of the cell cycle[45]. Loss of ERβ expression is considered to be a common feature in estrogen-dependent breast tumor progression[34,35] supporting the hypothesis that ERβ acts as a protector against the mitogenic activity of estrogen in breast pre-malignant tissues.

Estrogen is also critical for the progression of ovarian cancer[46-48]. A strong association between long-term estrogen replacement therapy and increased risk of ovarian cancer has been detected in several studies[45-47]. Similar to breast cancer, the imbalance between ERα and ERβ, along with decreasing expression of ERβ in the ovaries can also lead to uncontrolled cellular proliferation, subsequent malignancy and metastasis[49,50]. Thus, ERβ appears to be pro-apoptotic, facilitating the destruction of malignant cells, whereas ERα has anti-apoptotic activity, indicating its growth stimulatory role[34,45,49]. Confirming the role of ERβ as a tumor-suppressor, deletion of chromosome 14q, where ERβ co-localizes with some other tumor suppressors, is often detected in breast, colon, ovarian and prostate malignant tissue[51-54].

In contrast to the cancer-promoting role of estrogen in breast and ovarian cancers, it has been shown that estrogen works as a cancer suppressor for several gastrointestinal malignancies[41,42,44-56]. The Women's Health Initiative study, which included a cohort of 16 608 women randomized to hormone replacement therapy (HRT) *vs* no HRT, showed that the risk of colorectal cancer was almost halved in women using HRT[55]. A similar study in the United Kingdom of patients with esophageal and gastric cancer concluded that HRT was associated with a 50% reduction in the risk of gastric and colon adenocarcinoma, but had no significant benefit for esophageal adenocarcinoma[56]. However, due to the relatively small number of females with esophageal adenocarcinoma in this study (n = 299), the power of the study was limited and the question remains, thus, unresolved[41,42].

The male predominance of approximately 2:1 in gastric cancer incidence across the world cannot be explained on the basis of gender differences for the prevalence of known risk factors[57]. It has been hypothesized that estrogens play a protective role against gastric cancer. This statement has gained further support from a clinical study of a male cohort of patients with prostate cancer. In this study the risk of developing gastric cancer was lower amongst those who had been treated with estrogen than in those without such treatment (standardized incidence ratio, 0.87; 95% confidence interval, 0.78-0.98)[58]. Further supporting this argument are studies which have shown decreased ERβ expression in other gastrointestinal cancers, such as colon cancer, compared to benign tumors and normal tissues[59]. Tamoxifen exposure has also been shown to be a risk factor for gastric cancer[60,61], adding support to the idea that estrogen signaling has a protective role against gastrointestinal cancer.

FAT DISTRIBUTION, LEPTIN AND ESTROGEN: IS THERE A LINK?

There is a growing appreciation that estrogens are not only directly involved in the reproductive process and in regulation of carcinogenesis, but also have general metabolic roles in both sexes[15-17]. Estrogen signaling has a complex relationship with obesity that differs for premenopausal and postmenopausal women[12]. Importantly, obesity is a risk factor for esophageal adenocarcinoma in both women[62] and men[63]. In a recent study of 23 women with esophageal adenocarcinoma[63], 21 (91.3%) were in the top half of the distribution of the studied cohort with regard to waist-to-hip ratio, waist circumference, and body mass index. Multiple studies of male cohorts have demonstrated a strong association between increased abdominal diameter and esophageal adenocarcinoma, after controlling for body mass index and gastroesophageal reflux[63-68]. It is possible that associations between obesity and esophageal cancer are similar for both sexes, even though the regulation of adiposity in men and women differs significantly. For instance, distribution of body fat in men is characterized by the accumulation of visceral fat, but in women by subcutaneous fat.

Subcutaneous and visceral fat tissues express variable levels of both types of ER[69-71]. However, only ERα has a significant influence on energy homeostasis. The role of ERα in estradiol regulation of body weight and obesity is supported by the following observations: (1) both male and female mice that have been genetically altered to reduce the ability to produce estrogen by knocking out aromatase (an enzyme that catalyzes the conversion of androgen to estrogen) became obese when fed the same amounts as normal mice[72]; and (2) increased white adipose tissue and body fat were seen in both sexually mature male and female ERα-knockout mice[73,74]. Further supporting a role for estrogen signaling through ERα in the regulation of body weight are the findings that abnormal adiposity has been associated with the XbaI polymorphism of the human ERα gene[75,76].

The role of ERβ in estradiol regulation of body weight and obesity is less clear and somewhat controversial suggesting that ERβ functions more as a modulator of estrogen actions[71].

Estrogen has also been shown to contribute to the regulation of body adiposity and fat distribution through ERs in the brain[77], and by interacting with leptin signaling pathways[78]. 17β-estradiol increases leptin mRNA levels in adipose tissue[79]. Consistently, estrogen deficiency impairs central leptin sensitivity[77,78]. In women, leptin fluctuations during the menstrual cycle correlate directly with secretion of estrogen[79,80]. Estrogen has also been found to influence leptin receptor expression and hypothalamic sensitivity to leptin driving subcutaneous body fat accrual over visceral fat during the estrous cycle in rats[81]. Hence, visceral fat varies inversely with estrogen levels. Visceral fat accumulates in females when circulating estrogen levels become sufficiently low, as in postmenopausal women[76,78,82]. The accumulation of visceral fat is associated with an increased risk of various gastrointestinal malignancies, including esophageal adenocarcinoma[83]. Thus, estrogen regulation of leptin levels in women may play a protective role, directing accumulation of subcutaneous in preference to visceral fat.

The situation for men, however, is less clear, although a high level of leptin is considered to be a risk factor for males to develop esophageal adenocarcinoma[63,83]. Speculatively, the production of, and sensitivity to, leptin in men may be increased in visceral fat, and locally in tissues located in close proximity to adipose tissue where estrogen synthesis may be increased. However, mechanisms of ER and leptin signaling in males remain obscure, mostly because the majority of laboratory findings and clinical investigations of leptin and estrogen signaling have used tissues from females. To address this issue, studies are needed that specifically address the role of estrogen signaling in male adipose tissue.

IMPLICATIONS OF ESTROGEN RECEPTOR EXPRESSION IN ESOPHAGEAL ADENOCARCINOMA

In 1998, Lagergren *et al*[83] hypothesized that high estrogen and/or progesterone levels, low testosterone, or a combination of both, might contribute to the lower incidence of esophageal carcinoma in women. Epidemiological data for esophageal adenocarcinoma demonstrates a profound gender difference, with the male: female ratio exceeding 8:1, strongly supporting this hypothesis[1-4]. There are no detailed studies that compare the expression of ERs in esophageal tissues between males and females, but a limited number of studies have provided some preliminary data comparing ER expression in esophageal adenocarcinoma and its precursor lesion, Barrett's esophagus. These studies are summarized in Table 1.

In contrast to the anti-tumor role of ERβ in other cancers, some studies have identified a positive association between ERβ expression and esophageal adenocarcinoma development. Akgun *et al*[84] determined ERβ expression in the esophageal mucosa from patients with Barrett's metaplasia negative for dysplasia, Barrett's metaplasia with low grade dysplasia and Barrett's metaplasia with high grade dysplasia. The results of this study showed significant expression of ERβ (more than 50% of cells positive) in all patients with esophageal adenocarcinoma, and there was a trend towards increased expression of ERβ as the esophageal lesions progressed[85]. These results raise the possibility of ERβ as a target of therapy for esophageal adenocarcinoma. Similarly, another investigation showed a moderate increase in ER expression in tissue samples from men and women with Barrett's esophagus and esophageal adenocarcinoma. However, the subtype of ER was not determined in this study[39].

As ERβ has several isoforms, and these isoforms

Table 1 Estrogen receptor expression in patients with esophageal adenocarcinoma

	No. of patients with EAC	ERα	ERβ	Conclusion
Akgun *et al*[84]	31	Not expressed	Increased expression as esophageal lesions progressed	ERβ is suggested as a EAC therapy target
Tiffin *et al*[85]	20 (8)	Type of ER was not specified; ER were detected in EAC patients		ER may be important for further investigation
Liu *et al*[39]	33	Not expressed	Expressed in EAC, but not in Barrett's esophagus	Anti-estrogen treatment could be a promising therapeutic target for EAC
Kalayaransan *et al*[86]	45 (15)	Not expressed	Detected in all 45 patients; Expressed higher in EAC, compared to normal esophageal mucosa	ERβ suggested as marker and/or prognostic factor

EAC: Esophageal adenocarcinoma; ER: Estrogen receptor.

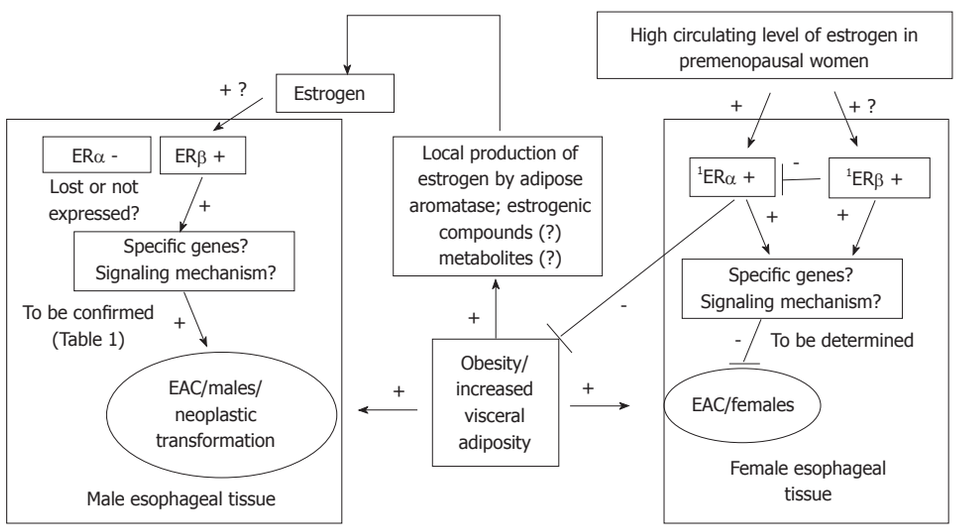

Figure 1 Role of estrogen-activated signaling in the development of esophageal adenocarcinoma: hypothetical pathway. [1]Expression level of estrogen receptor (ER) needs to be determined in esophageal tissue from females and compared to expression in males. EAC: Esophageal adenocarcinoma.

have different functions, Liu *et al*[39] identified which isoforms of ERβ were expressed in esophageal adenocarcinoma but not in Barrett's esophagus. All isoforms of ERβ showed much higher expression in esophageal adenocarcinoma, than in its precursor lesion, Barrett' s esophagus. Thus, a possible role for ERβ isoforms in the maintenance and evolution of esophageal adenocarcinoma was suggested. Although the study did not find a correlation between immunoreactivity and cancer proliferative activity, it showed that ERβ1 tended to have higher expression in invasive tumors which had penetrated the full thickness of the esophageal wall, compared to tumors limited to the esophageal wall ($P = 0.05$), and ERβ1 immunostaining tended to be most prominent in invasive esophageal adenocarcinoma. Conclusively, the study detected the presence of ERβ isoforms in esophageal adenocarcinoma and suggested the potential use of anti-estrogen treatment as a therapeutic target for esophageal adenocarcinoma. Manipulation of ERβ signaling may be considered as a potential prevention strategy to delay or block progression from dysplasia to esophageal adenocarcinoma. Figure 1 summarizes a potential mechanism for interaction between estrogen, ERs and esophageal adenocarcinoma.

Another study by Kalayaransan *et al*[86] determined the expression of ERα and ERβ in esophageal adenocarcinoma across various classifications of tumor stage, and compared expression with adjacent normal esophageal mucosa. No significant expression levels of ERα were found in esophageal adenocarcinoma, suggesting ERα is unlikely to mediate the growth of esophageal adenocarcinoma. However, immunostaining with ERβ antibodies yielded significantly higher results in esophageal adenocarcinoma, compared to normal esophageal mucosa[87]. In each group with the same degree of tumor differentiation, tumor samples had significantly higher staining scores compared to normal esophageal mucosa. Tumors with good or moderate differentiation had lower staining scores than those which were poorly differentiated, indicating that the potential effect of estrogen on esophageal adenocarcinoma could be mediated by ERβ[84,86,87]. Overall, most studies that have evaluated esophageal adenocarcinoma are consistent in suggesting a detrimental effect and prognostic value for ERβ.

Unfortunately, these clinical findings have not yet been supported by *in vitro* experiments using esopha-

geal adenocarcinoma cells. The few *in vitro* studies that have addressed the role of estrogen in the regulation of esophageal cell growth were conducted using squamous cancer cells[87,88]. It has been shown that the growth of an ER-positive esophageal squamous carcinoma cell line (ES-25C) is significantly inhibited by 17β-estradiol, whereas this effect is not observed in an ER-negative squamous carcinoma cell line (ES-8C)[87]. A similar finding was seen in another study, in which the proliferation of the ER-positive KSE-1 esophageal squamous carcinoma cell line was inhibited by 17β-estradiol[88]. In addition, *in vivo* growth of this cell line in both female and male mice was suppressed by the administration of 17β-estradiol, raising the possibility of manipulating the growth of esophageal carcinoma by manipulating the estrogen-ER system[88]. However, esophageal squamous cell carcinoma and esophageal adenocarcinoma are two biologically distinct diseases, so estrogen responsiveness in squamous cell carcinoma lines does not automatically mean that esophageal adenocarcinoma cell lines will also respond. Similar experiments need to be performed on esophageal adenocarcinoma cell lines in order to explore this possibility further.

FUTURE PERSPECTIVES

Current literature provides only limited evidence for a link between estrogen and the development of esophageal adenocarcinoma. Hence, a series of questions can be proposed, and further studies will be needed to determine whether there is any link. It is unclear whether there is a gender difference for the expression of ERβ, or correlation between tumor stage and the expression of ERβ. Most previous studies have not compared estrogen effects in both genders, and have only addressed men and women separately. Detailed comparisons have not been done for various esophageal pathologies *vs* normal esophageal mucosa within both gender groups. Another limitation of previous studies is the small number of patients studied, and for this reason reported data is yet to be verified. A systematic study which includes a sufficiently large number of men and women is needed to determine whether, within each gender group, ERβ expression is associated with the development and progression of esophageal adenocarcinoma. A confirmed link might provide support for ERβ to be used as a target for therapy, or as a prognostic marker.

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TOPIC HIGHLIGHT

Anastasios Koulaouzidis, MD, MRCP, *Series Editor*

Update of endoscopy in liver disease: More than just treating varices

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Abstract

The management of complications in liver disease is often complex and challenging. Endoscopy has undergone a period of rapid expansion with numerous novel and specialized endoscopic modalities that are of increasing value in the investigation and management of the patient with liver disease. In this review, relevant literature search and expert opinions have been used to provide a brief overview and update of the current endoscopic management of patients with liver disease and portal hypertension. The main areas covered are safety of endoscopy in patients with liver disease, the use of standard endoscopy for the treatment of varices and the role of new endoscopic modalities such as endoscopic ultrasound, esophageal capsule, argon plasma coagulation, spyglass and endomicroscopy in the investigation and treatment of liver-related gastrointestinal and biliary pathology. It is clear that the role of the endoscopy in liver disease is well beyond that of just treating varices. As the technology in endoscopy expands, so does the role of the endoscopist in liver disease.

INTRODUCTION

Liver disease and cirrhosis are common causes of mortality worldwide[1]. The role of endoscopy in liver disease is both diagnostic and interventional: endoscopy should be offered to patients with relevant symptoms (unsuspected liver disease may be diagnosed in this manner) and for variceal screening and treatment. Patients with liver disease can be challenging to sedate, and the complexity of endoscopy in liver disease continues to increase with rising numbers of patients with a liver transplant, and the advent of new endoscopic modalities such as capsule endoscopy and endoscopic ultrasound (EUS).

SEDATION AND ANALGESIA IN LIVER DISEASE

Pharmacodynamics are altered in advanced liver disease as a result of changes in hepatic conjugation and oxidation, shunting, decreased protein binding and an increased vol-

ume of distribution[2]. The common agents used for sedation in endoscopy are discussed, however, specific doses cannot be recommended because these are dependent on patient factors. We would recommend that an endoscopist or anesthetist who has experience with this patient group undertakes the sedation of liver patients.

Benzodiazepines

Midazolam is the benzodiazepine of choice in most endoscopy units. It is protein bound and metabolized in the liver by cytochrome P3A4. In cirrhosis, clearance of midazolam is impaired and elimination half-life is doubled. As a result, midazolam should be used with caution in patients with cirrhosis[3].

Opiates

Pethidine and fentanyl are the most commonly used analgesics for endoscopic procedures. The liver is the major site of biotransformation for most opiates. The oxidation of pethidine is reduced in patients with cirrhosis and its clearance is diminished. Therefore, there is increased bioavailability, and pethidine should be avoided in patients with liver disease[4]. The half-life of fentanyl is shorter and does not seem to be influenced by cirrhosis. Its use is preferred to pethidine[5].

Anesthetic agents

The pharmacokinetics of propofol, an anesthetic agent that is widely used in endoscopy, appears to be unaffected by cirrhosis; again, perhaps secondary to its short half-life. One study has suggested that the use of propofol rather than midazolam in patients with compensated cirrhosis facilitates a faster recovery time with less exacerbation of subclinical encephalopathy[6].

Endotracheal intubation

Gastrointestinal (GI) bleeding in patients with liver disease may be life threatening. If bleeding varices are suspected or patients are hemodynamically unstable, there is often a low threshold for endotracheal intubation to protect the airway. There is little literature on prophylactic intubation for airway protection in such patients, and two retrospective studies[7,8] have concluded that it does not prevent cardiopulmonary complications, or pneumonia. We are of the opinion that airway protection at emergency endoscopy is extremely important in patients with suspected variceal bleeding, who present with hematemesis, and particularly in hemodynamically unstable patients and those with hepatic encephalopathy or alcohol withdrawal symptoms. In such patients, endoscopy is best undertaken in a critical care environment with immediate access to anesthetic support and endotracheal intubation[9].

ENDOSCOPY IN THE CIRRHOTIC PATIENTS WITH COAGULATION ABNORMALITIES

Coagulopathy and thrombocytopenia are common in patients with chronic liver disease. The mechanisms behind coagulation abnormalities are often complex, and it is now thought that prolongation of prothrombin time may not directly relate to the risk of bleeding, and rather, it is the balance of pro- and antithrombotic factors that is important. In practical terms, there is currently no reliable way of quantifying this. Routine correction of coagulopathy at endoscopy is not recommended, although patients with chronic liver disease should receive vitamin K to correct any dietary deficiency that may result in coagulopathy.

It is recognized that diagnostic endoscopy is a low-risk procedure and safe in patients with altered coagulation. However, high-risk endoscopic therapeutic procedures have a significantly increased risk of hemorrhage and, as such, coagulopathy should be treated[10]. It is therefore common practice in cirrhotic patients to correct significant thrombocytopenia ($< 50 \times 10^6$/mL) with platelet transfusions and to correct coagulopathy with fresh frozen plasma (FFP) if prothrombin time is > 20 s to an international normalized ratio < 1.5, before high-risk procedures. Platelet and FFP transfusions are particularly helpful during an acute bleeding episode if the prothrombin time is prolonged or platelets are low, similar to the previously mentioned values[11].

Novel treatments include recombinant factor Ⅶa. This has been used as a hemostatic agent in acute variceal bleeding, but failed to show efficacy in a large randomized study[12]. Another trial has demonstrated that addition of desmopressin does not improve and may worsen the efficacy of terlipressin in controlling acute variceal bleeding in cirrhotic patients[13].

Certain endoscopic investigations have been shown to be safe in coagulopathic patients with cirrhosis, despite being relatively invasive; EUS-fine needle aspiration of the liver has been shown to be a safe alternative to percutaneous liver biopsy, particularly in patients with advanced liver disease, coagulopathy and high risk of bleeding[14]. At endoscopic retrograde cholangiopancreatography (ERCP), endoscopic papillary balloon dilation is safer than endoscopic biliary sphincterotomy for the treatment of choledocholithiasis in patients with advanced cirrhosis and coagulopathy, because it has a reduced risk of bleeding[15].

COMMON ENDOSCOPIC DIAGNOSES AND MANAGEMENT IN PATIENTS WITH LIVER DISEASE

Peptic ulcer disease

The correlation between peptic ulcer disease and cirrhosis is well described. Both duodenal and gastric ulcers are more common in cirrhosis: the reported prevalence is 24.1%[16]. It is recognized that the prevalence of gastric ulceration increases with the severity of liver disease and is related to changes in the hepatic venous pressure gradient[16,17].

A high prevalence of *Helicobacter pylori* (*H. pylori*), up

Table 1 Summary of primary prophylaxis of esophageal varices

Grade	Appearance	High-risk stigmata	Treatment
Grade 1: Small varices	Barely noticeable varices; disappear easily with insufflation	No red signs	No treatment
Grade 2: Small/medium varices	Small or medium varices; do not easily disappear with insufflation	± Red signs	NSBB or VBL
Grade 3: Medium/large varices	Medium or large varies; do not disappear with insufflation	± Red signs	NSBB or VBL

NSBB: Non-selective b blockers; VBL: Variceal band ligation.

to 89%, in patients with cirrhosis has been reported[18]. 13C urea breath testing and gastric body histology remain highly accurate in detecting *H. pylori* in cirrhosis, whereas rapid urease tests and serology are less reliable than in non-cirrhotic patients[19]. A meta-analysis of seven studies with almost 1000 patients has strongly suggested that, as with non-cirrhotic patients, *H. pylori* infection increases the risk for peptic ulcer disease in cirrhosis[20]. *H. pylori* eradication therapy is effective in chronic liver disease[21]. However, two recent studies have suggested that *H. pylori* eradication in cirrhotic patients with duodenal ulcers is not as effective at reducing ulcer recurrence as it is in the general population. These patients require maintenance acid suppression therapy[22,23].

Portal hypertension

The development of portal hypertension and formation of portosystemic shunts is a major event in the natural history of liver disease. Measurement of the portal pressure gradient is invasive and not widely available for clinical use; instead the hepatic venous pressure gradient (HVPG) is commonly used in clinical practice and it is of prognostic value: HPVG $\geqslant$ 10 mmHg strongly predicts the development of esophageal varices[24]. Similarly, the most significant risk factor associated with failure to control bleeding or early rebleeding of esophageal varices is HVPG > 20 mmHg. This is also associated with increased mortality[25].

Gastroesophageal varices are present in > 50% of patients with portal hypertension and are more likely as liver disease progresses[26]. Ectopic varices are located in sites other than the gastroesophageal region and are more common than previously thought: duodenal or colonic varices are seen at angiography or colonoscopy in up to 40% of patients with intrahepatic portal hypertension[27].

Esophageal varices

It is recommended that all patients undergo endoscopy to assess the presence and the size of varices at the time of the diagnosis of cirrhosis. Thereafter, guidelines for the interval of endoscopic screening vary. Currently, the American Association for the Study of the Liver (AASLD) recommends that, if no varices are present at index endoscopy, this should be repeated at 2-3 years in compensated cirrhosis and annually in decompensated cirrhosis[11]. The British Society of Gastroenterology recommends annual screening if grade 1 varices are present at initial screening (Table 1, grading and treatment of esophageal varices), and an interval of 3 years if there is no evidence of varices at index endoscopy[28].

Esophageal variceal bleeding occurs at a rate of 5%-15% per year in untreated patients. The main risk factors for bleeding are variceal size (grade 2 or 3), decompensated cirrhosis, and the presence of high-risk stigmata at endoscopy[29]. Variceal bleeding is a significant clinical event with a mortality rate of approximately 20% at 6 wk, and a recurrence rate of up to 60% at 2 years if secondary prophylaxis is not commenced[30].

The management of esophageal varices may be divided into pre-primary, primary and secondary prophylaxis and control of active bleeding. At present, there is no evidence to support treatment to prevent the development of varices in patients with liver disease (pre-primary prophylaxis)[31,24].

For primary prophylaxis of esophageal varices, there is no evidence that variceal band ligation (VBL) is superior to β-blockade. Due to issues with access to endoscopy and patient preference, non-selective β-blockade, typically with propranolol, is often first line when treatment is indicated[32]. Carvedilol is a potent non-selective β-blocker, with weak vasodilating properties. A reduction in the HVPG in the range of 10%-43% has been reported with a 12.5 mg/d dose. Carvedilol has therefore been adopted as the β-blocker of choice for primary prophylaxis of variceal bleeding in some centers[33-35]. Primary prophylaxis with VBL is recommended if there are contraindications to β-blockers, or concerns about patient compliance[11].

Secondary prophylaxis is indicated for patients who have had an episode of variceal hemorrhage. β-blocker monotherapy is not used as secondary prophylaxis, and patients should either be entered into a variceal banding program or receive a combination of a β-blocker and nitrate[36]. AASLD recommends a combination of β blockade and VBL[11]. However, there is no strong evidence to suggest that this strategy is associated with improved mortality[37] and our local practice is to use VBL alone. VBL should be repeated every 2 wk until obliteration of varices is achieved. Following this, a surveillance endoscopy at 1-3 mo to confirm eradication is required, and this should be repeated every 6-12 mo[11]. VBL is a safe technique: although asymptomatic banding ulcers are common after VBL, the rate of bleeding from these and requiring hospitalization does not exceed 5%[38].

The combination of terlipressin and VBL is the preferred treatment for acute variceal bleeding in many centers, and using terlipressin before endoscopy is not unreasonable if there is a delay to the endoscopy. Endo-

scopic hemostasis is usually achieved in the majority of cases[11]. Transjugular intrahepatic portal systemic shunt (TIPSS) may be considered if VBL has been unsuccessful or there is an early re-bleed (defined at Baveno V as a repeat bleed within 5 d of the index bleed). A reduction in HVPG below 12 mmHg or a 20% reduction from the baseline value, even without reaching < 12 mmHg, protects against rebleeding[39].

The use of sclerosing agents (variceal sclerotherapy) is no longer recommended as first-line treatment, because of increased mortality rates[40], nor for secondary prophylaxis because VBL treatment has been shown to be safer and more effective[41].

Recently, endoscopic placement of a specifically designed self-expanding covered metal stent has proved effective in the treatment of esophageal varices in patients in whom initial endoscopic methods have failed to achieve hemostasis[42]. This method appears to be a safe and effective means of controlling ongoing bleeding. The stent is usually removed 1 wk after the acute bleed. Currently, this technique is limited by its relative complexity of stent insertion in acute bleeding, but stenting with covered biodegradable stents when available, which do not require removal, may play an important role in the management of acute esophageal variceal bleeding[43].

Gastric and ectopic varices

Gastric varices are less prevalent than esophageal varices and less prone to bleeding: around 25% over a 2-year period[44]. There is no evidence to support the primary prophylaxis of gastric varices. The tissue adhesive cyanoacrylate ("glue") is used widely in the management of acutely bleeding gastric varices. Cyanoacrylate is a liquid with consistency similar to water, which when added to a physiological fluid like blood, polymerizes to form a solid substance[45]. Two randomized controlled studies have compared cyanoacrylate with VBL for management of bleeding gastric varices[46,47]. In one study, cyanoacrylate was more effective than VBL in achieving homeostasis, and in the second, no difference was reported, although both reported less recurrence of bleeding. Current evidence suggests that cyanoacrylate achieves control of bleeding in 87%-93% of cases, and that bleeding-related mortality is between 6.5% and 10%[46,47].

The use of bovine or more recently, human thrombin has been described as an alternative treatment for active gastric variceal bleeding. Thrombin [activated factor Ⅱ (Ⅱa)] is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin clot. It has additional effects including promotion of platelet aggregation. Initial hemostasis rates have been reported at 94%-100%, and rebleeding rates of between 23% and 25%[48,49]. A single center experience of 13 patients treated with thrombin for bleeding gastric varices has reported efficient hemostasis and an overall mortality of 38% in a median follow-up of 22 mo[50].

Technical difficulties that include the risk of equipment damage and reports of severe thromboembolic complications may limit the use of cyanoacrylate, and thrombin (Figure 1) may become more widespread in the future[51,52].

The role of TIPSS as primary treatment in actively bleeding gastric varices has also been explored. Although TIPSS has a comparable mortality and rebleeding rate to cyanoacrylate, it is associated with significantly higher morbidity and is not used as first-line treatment[53,54], but remains an effective treatment when endoscopy fails to control bleeding.

Nonvariceal manifestations of portal hypertension

Portal hypertensive gastropathy (PHG), with its typical "snake skin" appearance, is present in approximately 80% of patients with cirrhosis[55†]. PHG accounts for 8% of nonvariceal bleeds in patients with liver disease, although this condition more commonly presents with anemia[56]. Patients with cirrhosis and severe PHG-related bleeding may respond to β-blockade. Endoscopic measures such as argon plasma coagulation (APC) therapy can reduce bleeding, thus controlling anemia. TIPSS should be reserved for those patients with pharmacological treatment failure[57].

The prevalence of portal hypertensive enteropathy (PHE), determined by capsule endoscopy, is as high as 63% in patients with end-stage liver disease who also have esophageal or gastric varices[58]. Portal hypertensive duodenopathy is present in around half of patients with cirrhosis, and it is more common in patients with severe PHG[59].

Gastric antral vascular ectasia

Gastric antral vascular ectasia (GAVE) is related to portal hypertension in about 30% of patients, and accounts for 4% of nonvariceal upper GI bleeds[60]. Unlike PHG, GAVE does not respond well to reduction in portal pressure[61]. The Nd:YAG laser has been widely used in the treatment of GAVE and is the most commonly reported endoscopic modality in cirrhotic and non-cirrhotic patients, with a reported overall success rate of almost 90%, although the authors did not distinguish the etiology of GAVE when reporting the outcomes[62].

APC has also proved to be effective for the treatment of GAVE-related bleeding, and it is extensively used in our unit, with a success rate of > 85%; success is defined as control of bleeding, stabilization of hemoglobin at > 100 g/dL, or hemoglobin increase > 10% from pretreatment level, and reduction of transfusion requirements by > 50% in transfusion-dependent patients. An average of four sessions of APC is usually required (Figures 2 and 3). In two published studies, with a total of 37 patients with cirrhosis, success rates in controlling bleeding were very high, and the reported rebleeding rates were between 12% and 20% after 2 years follow-up[62,63].

Although the success rates of the two aforementioned modalities are comparable, APC treatment is probably the therapy of choice due to the technical ease, safety and low cost[61]. Other endoscopic techniques have been positively reported in small series, such as VBL[64],

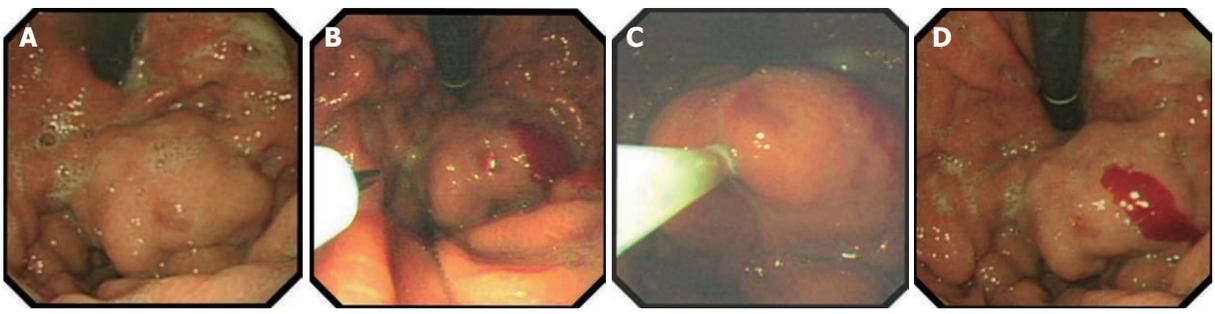

Figure 1 Endoscopic images of fundic gastric varices before (A), during (B, C) and after (D) thrombin injection.

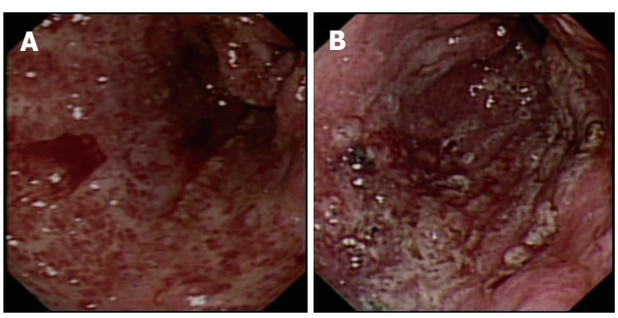

Figure 2 Endoscopic image. A: Gastric antral vascular ectasia (GAVE) (diffuse type) with active bleeding prior to argon plasma coagulation (APC) treatment; B: GAVE (diffuse type) immediately after APC treatment.

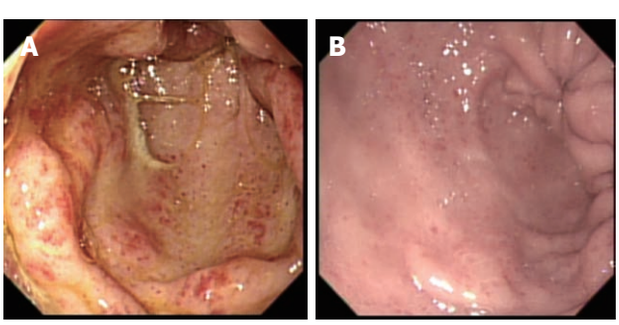

Figure 3 Endoscopic image. A: Gastric antral vascular ectasia-induced symptomatic anemia; B: Endoscopic image of the same patient 2 years later, after several argon plasma coagulation sessions. The number of angioectatic lesions in the gastric outlet had dramatically decreased.

endoscopic mucosal ablation[65] and cryotherapy[66].

ADVANCED ENDOSCOPIC PROCEDURES AND THEIR VALUE IN LIVER DISEASE

EUS

Recently, EUS has been used to assist in the management of portal hypertension. Doppler EUS is of significant value in differentiating ectopic varices from other submucosal lesions[67] (Figure 4), and several EUS-assisted techniques have been used to identify the precise site for the intravariceal injection of sclerosant agents. Linear color EUS-guided sclerotherapy has proved to be effective in the eradication of esophageal varices, in two small studies with recurrence reported at 0% and 8.3%, respectively[68,69]. EUS catheter probes and high-frequency (20 MHz) miniprobes have both been used successfully before and after esophageal variceal sclerosant injection in two different studies to assess eradication and variceal recurrence. After a mean follow-up of 24 mo, variceal recurrence was reported at 16.6% and 26.3%, respectively[70,71].

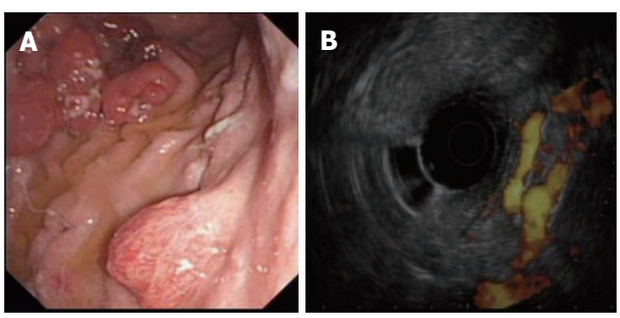

Figure 4 Endoscopic image of gastric irregular submucosal lesion. A: Gastric irregular submucosal lesion in a patient with portal hypertension; B: The same lesion examined under color Doppler endoscopic ultrasound. The submucosal lesion was hypervascular and represented a gastric varix.

Although the overall patient numbers are small, linear EUS seems to be the superior modality in assisting treatment of esophageal varices, because it permits the targeting of the feeding vessels.

EUS-assisted injection of cyanoacrylate for the treatment of gastric varices has been described in 54 patients with a mean follow-up of 24 mo. Varices recurred in 35% of patients[72]. Furthermore, a series of 15 patients with gastric or ectopic varices treated with thrombin injection in conjunction with a variety of EUS techniques (Figure 5) has recently been reported in our unit, and this proved to be effective in controlling active bleeding and achieving variceal eradication[73].

Capsule endoscopy

Esophageal capsule endoscopy (OCE) is an alternative to conventional upper GI endoscopy for the diagnosis of varices in complex patients with portal hypertension. In a recent meta-analysis of seven studies involving 446 patients, OCE had a sensitivity of 85.8% and specificity 80.5% in detecting esophageal varices[74]. However, a multicenter trial evaluating the efficacy of OCE in esophageal varices screening was less encouraging, because the standard of < 10% difference between capsule

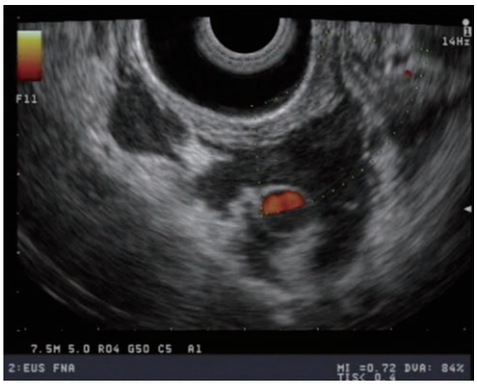

Figure 5 Color doppler endoscopic ultrasound image of duodenal varices after thrombin injection. The absence of blood flow and the speckled appearances were suggestive of thrombus formation.

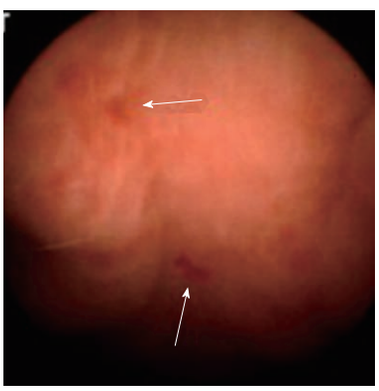

Figure 6 Small bowel capsule image of portal hypertensive enteropathy and stigmata of recent bleeding. Engorged small bowel villi and micro-hemorrhagic spots were visible.

and conventional endoscopy was not met[75]. This is not surprising, because the two techniques differ in that during conventional endoscopy, the esophagus is inevitably insufflated with air and varices can appear more flattened than during OCE examination. Further studies to take this into account are necessary. Nevertheless, OCE remains a useful tool for screening of varices in certain patient groups; patients who poorly tolerate endoscopy or who have significant comorbidity, thus increasing the risks of repeated endoscopy, and patients with high risk of variant Creutzfeldt-Jakob disease. Although this technique is limited by availability and high costs, OCE can be cost-effective for variceal screening of patients with coagulation abnormalities (e.g., hemophilia) with coexisting liver disease, because it does not require prophylactic clotting factor administration, unlike conventional endoscopy. Serial capsule examinations in the same patient may provide significant diagnostic information regarding progression of varices.

Small bowel capsule endoscopy (SBCE) has been used to characterize PHE (Figure 6), and is of value in the diagnosis of this condition in patients with advanced liver disease who continue to bleed despite treatment of esophageal/gastric varices or portal gastropathy (Figure 7).

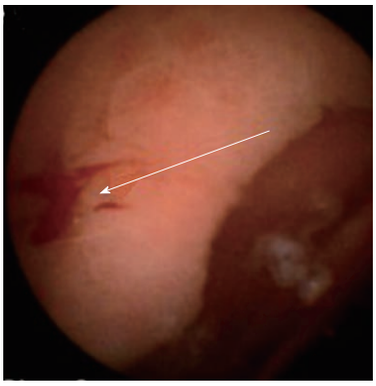

Figure 7 Small bowel capsule image of portal hypertensive enteropathy with snake-skin-like appearance of the mucosa and red spots as stigmata of recent bleeding.

The role of SBCE in portal hypertension has yet to be defined, but it is likely that it will remain a valuable tool in certain groups of patients with liver disease.

Endoscopic retrograde cholangiography

Primary sclerosing cholangitis (PSC) is characterized by fibrosis of both intrahepatic and/or extrahepatic biliary ducts. These patients are at risk of developing infectious cholangitis and up to 20% develop cholangiocarcinoma. A strategy of initial magnetic resonance cholangiopancreatography (MRCP) followed, if necessary, by ERCP is currently the most cost-effective approach to the work-up of patients with suspected sclerosing cholangitis[76†]. ERCP plus diagnostic brushing have a sensitivity of 60%-100%, and specificity of 85%-89% in differentiating between a benign dominant stricture and cholangiocarcinoma[77,78]. Recently, two advanced cytological techniques (digital image analysis and fluorescence *in situ* hybridization) have been used for the detection of malignancy in PSC-related strictures and have proved to be more sensitive and equally specific to conventional cytology[79†].

In addition, ERCP permits therapeutic interventions with balloon dilation or stent placement as appropriate.

Novel techniques and the biliary tree

Novel endoscopic modalities have been compared with conventional ERCP and brush cytology. Transpapillary cholangioscopy with tissue sampling has proved to be more sensitive (92% *vs* 66%) and specific (93% *vs* 51%) than ERCP to detect cholangiocarcinoma in PSC[80]. In a small study, narrow band imaging has demonstrated superior visualization of biliary lesions compared with conventional white light imaging[81]. In another study, transpapillary intraductal ultrasound was superior to ERCP for the detection of cholangiocarcinoma in PSC in terms of sensitivity (87.5% *vs* 62.5%) and specificity (90.6% *vs* 53.1%)[82].

Spyglass™ is a new single-operator system used for the diagnosis of a variety of pancreatobiliary disorders, such as the definition of indeterminate strictures and filling defects prior to stone extraction[83]. Although the

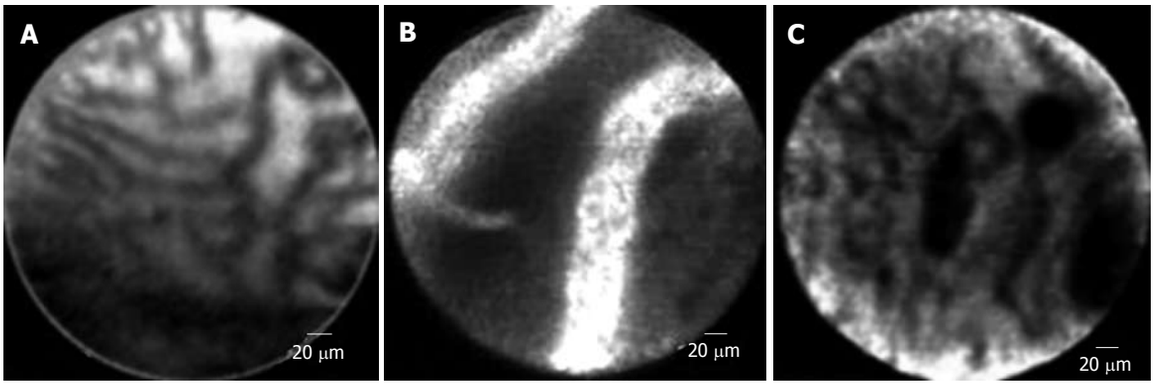

Figure 8 Endomicroscopy image. A: Image from Cellvizio® bile duct endomicroscopy. The regular reticular pattern of thin dark structures with low signal (dark) characterized the normal bile duct (Image courtesy of www. cellvizio.net); B: Abnormal bile duct appearances in Cellvizio® endomicroscopy; isolated blood vessels with very strong signal (with strands) suggestive of tumor neovascularization of cholangiocarcinoma (Image courtesy of www.cellvizio.net); C: Reticular pattern of dark bands and dark clumps or glands suggestive of cholangiocarcinoma (Image courtesy of www.cellvizio.net).

initial experience is promising, the modality has been only been tested in a limited number of PSC-related strictures, and unlike ERCP, this is a purely diagnostic technique.

Confocal laser endomicroscopy ("miniprobe") is a new field of endoluminal imaging that offers extremely high magnification and resolution. This technique allows visualization of pancreatic and biliary ducts. In a pilot study of 14 patients with biliary strictures, miniprobe-based microscopy after fluorescein administration proved to be more accurate than brushings and biopsy in distinguishing benign from malignant strictures (Figure 8)[84].

A novel alternative is direct cholangioscopy using ultra-slim endoscopes (4.9-5.9 mm). These endoscopes, initially developed for transnasal endoscopy, can be safely inserted into the bile duct following sphincterotomy, and not only permit high-resolution images, but also biopsy and other interventional procedures in the bile ducts, such as hydraulic lithotripsy and division of strictures in benign biliary disease. This technique is currently under development and after a full range of endoscopic accessories are available for endobiliary interventions, it could be an effective and safe approach for patients with difficult to manage biliary disease[85].

Endoscopic modalities in the lower GI tract

In comparison to the upper GI tract, colonic manifestations of portal hypertension much less often present with acute bleeding, and are more often found incidentally or during investigation of anemia. As such, data are sparse and less consistent. The reported prevalence of portal hypertensive colonopathy is 24%[86].

The most significant feature of portal hypertension in the colon is arguably the presence of rectal varices. These can be present in up to 44% of patients with cirrhosis at colonoscopy, although the reported prevalence varies widely. They are more frequent in patients with advanced liver disease[87].

Although bleeding from rectal varices is uncommon, it can be life threatening. Due to their rarity, no firm guidelines have been established for the management of bleeding colonic varices, and there is a limited evidence base. The most commonly used treatment modalities are sclerotherapy and band ligation. In a small retrospective comparative study of 15 patients, endoscopic injection sclerotherapy proved to be superior to endoscopic band ligation and achieved lower recurrence rates[88]. In our unit, thrombin has been successfully used to manage rectal variceal bleeding. Patients may require TIPSS if bleeding cannot be controlled endoscopically.

ENDOSCOPY AND THE LIVER TRANSPLANT PATIENT

Luminal diseases in liver transplant patients

Peptic ulcer disease is the most common cause of GI bleeding in post-orthotopic liver transplant (OLT) recipients, accounting for 27% of all bleeding[89]. Varices rarely recur post-transplant, and if present, require investigation to exclude portal vein thrombosis or disease recurrence[90].

Liver transplant patients are at increased risk of opportunistic infections, particularly candidiasis and cytomegalovirus[91]. These often present with GI symptoms and require endoscopic evaluation with biopsies or brushings to confirm the diagnosis.

The association of PSC with ulcerative colitis is well recognized and is an additional risk factor for the development of colorectal cancer in immunosuppressed transplant patients. It is currently recommended that these patients have an annual surveillance colonoscopy commencing 10 years after the onset of bowel symptoms[92]. Colectomy is safe in patients who have undergone OLT, and in some high-risk cases, such as when high-grade dysplasia has already been identified, a prophylactic colectomy may be performed at the time of transplantation[93].

Transplant-related biliary disease

Biliary complications (biliary strictures and leaks) following liver transplantation are a challenging and common

issue that affects 10%-30% of OLT patients. Biliary strictures are classified as anastomotic or non-anastomotic[94]. The initial approach in suspected post-transplant biliary strictures is usually MRCP, restricting the use of ERCP to patients who require intervention, or where MRCP results are equivocal[95]. Further imaging techniques include SpyGlass™, which has been successfully used for the investigation of post-transplant biliary strictures[96], and contrast-enhanced ultrasound. This is a non-invasive technique for the detection of strictures relating to hepatic artery stenosis in liver transplant patients. It provides details on the presence, location, degree, and type of stricture[97]. Treatment comprises a combination of balloon dilation and stent placement, repeated if necessary until stricture resolution.

Biliary leaks can occur in up to 22% of patients. Evidence suggests that sphincterotomy with stent placement is the best treatment option for biliary leaks following OLT[94,98]. Surgical revision and biliary reconstruction with the formation of hepaticojejunostomy is indicated when endoscopic or percutaneous treatment fails[94,98].

In patients who have a roux-en-Y anastomosis, the technique of double balloon ERCP has been devised with promising results. The technique uses a double balloon colonoscope to approach the ampulla, although there are some limitations regarding endoscopic accessories. With further development of this technique, the endoscopic treatment of biliary complications may become easier and will play a larger role in the management of such patients[99].

CONCLUSION

Endoscopy has undergone rapid expansion with numerous novel endoscopic modalities and techniques directly applicable to the diagnosis and management of complications of liver disease. Although conventional upper GI endoscopy is still the modality of choice for esophageal variceal surveillance and treatment, further options are now available with the use of capsule endoscopy and EUS. Therapeutic options for the management of upper GI bleeding in portal hypertension have also been developed. Band ligation remains the treatment of choice for esophageal variceal bleeding, whereas for gastric and ectopic varices, the use of sclerosants, particularly "glue" and thrombin are increasingly being used. APC is the preferred modality for GAVE and PHG. Biliary strictures and the risk of cholangiocarcinoma are major issues in patients with PSC. ERCP is both diagnostic and therapeutic in this setting and can differentiate benign from malignant lesions in the majority of cases. Novel endoscopic techniques such as transpapillary cholangioscopy, Spyglass Direct Visualization System, confocal laser endomicroscopy ("miniprobe") and ultra-thin cholangioscopy are increasingly being used to assist diagnosis in selected patients. Finally, in the post-liver transplant patient, upper and lower endoscopies are used to detect gastrointestinal opportunistic infections, as well as to screen for colorectal cancer in high-risk patients. Biliary complications are common after transplantation and ERCP is the modality of choice for treating such patients.

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REVIEW

Role of *ATG16L*, *NOD2* and *IL23R* in Crohn's disease pathogenesis

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Abstract

Inflammatory bowel disease is a group of diseases that includes Crohn's disease (CD) and ulcerative colitis. CD is characterized as a chronic inflammatory disease of the gastrointestinal tract, ranging from the mouth to the anus. Although there are gross pathological and histological similarities between CD and Johne's disease of cattle, the cause of CD remains controversial. It is vital to understand fully the cause of this disease because it affects approximately 500 000 people in North America and Europe. It ranges from 27 to 48 cases per 100 000 people. There are many theories on the cause of CD ranging from possible association with environmental factors including microorganisms to imbalance in the intestinal normal flora of the patients. Regardless of the environmental trigger, there is strong evidence that a genetic disposition is a major key in acquiring CD. Many studies have proven the link between mutations in the *ATG16L*, *NOD2/CARD15*, *IBD5*, *CTLA4*, *TNFSF15* and *IL23R* genes, and CD. The purpose of this review is to examine all genetic aspects and theories of CD, including up to date multiple population studies performed worldwide.

Key words: Crohn's disease; *ATG16L*; *NOD2/CARD15*; *IBD5*; *CTLA4*; *TNFSF15*; *IL23R*

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INTRODUCTION

The first description of Crohn's disease (CD) was made in 1769 by an Italian physician, Giovanni Battista Morgagni, when he diagnosed a man with chronic diarrhea. In 1898, consecutive cases were reported by John Berg, and then in 1904 by Antoni Lesniowski. Throughout the 1920s and 1930s, young adults were thought to have this same condition as they suffered from symptoms, such as abdominal cramps, diarrhea, fever and significant weight loss. In 1923, surgeons at Mt. Sinai Hospital in New York had also identified patients with comparable symptoms. In addition, in 1930, Dr. Burrill B Crohn saw a connection between this unknown debilitating condition and two of his patients. Consequently, Dr. Crohn and his

colleagues presented a paper in 1932, "Regional ileitis: a pathologic and chronic entity", describing the features of this disease to the American Medical Association. CD was named after Burrill Crohn and it became an official medical entity in 1932.

CD is a chronic inflammatory disease that can affect any portion of the digestive tract including the mouth, esophagus, and small and large intestines, but is most common in the ileum. It is also characterized as an autoimmune disease in which the body attacks itself and causes inflammation. In its mild form, it causes erosions called aphthous ulcers in the inner surface of the bowel. In severe cases, deeper and larger ulcers develop that can lead to bowel obstruction and holes in the bowel wall. If a hole in the bowel wall arises, infection to neighboring organs can occur. CD branches off to many forms depending on the location of erosion. If these symptoms take place within the large intestine, it is called Crohn's or granulomatous colitis. If it occurs in the small intestine, it is known as Crohn's enteritis, and more specifically if it occurs in the ileum, it is called Crohn's ileitis. During severe cases in which the small and large intestine are both involved, it is known as Crohn's enterocolitis or ileocolitis. CD is separated into three phenotypes: non-stricturing and non-penetrating, stricturing and penetrating CD[1,2].

CD is most prevalent in North America and Europe, and least prevalent among African Americans and Asians[3]. It affects approximately 500 000 people in North America and Europe. It ranges from 27 to 48 cases per 100 000 people. There is no difference in prevalence among males or females. Individuals with siblings affected by CD have a higher risk of acquiring the disease. There is evidence of a cause from environmental factors, thus, there are a higher number of cases in western industrialized countries. Symptoms of CD typically begin in the teens and twenties and then go into remission on and off with appropriate therapies. There is a peak incidence between 50 years and 70 years of age, which often leads to major complications due to age and the necessity of surgery.

Although the etiology is still unknown, there are many theories about what causes CD and ulcerative colitis (UC). Many believe it is caused by environmental factors, such as certain foods, bacteria, viruses, or cigarette smoke, which all can trigger an immune system response. Scientists have linked inflammatory bowel diseases (IBDs) as an autoimmune problem. In a healthy person, the immune system defends the body against harmful microbes that have entered it. Upon triggering the immune system, an inflammatory response occurs in which immune cells aggregate at the site of infection and overcome the threat. There are microbes native to our bodies that are useful rather than harmful to which the immune system does not trigger a response. In patients with CD, the immune system will attack these native luminal bacteria, disrupting the normal flora, thus characterizing this condition as an autoimmune disease.

Recent research has indicated specific genetic variations as a direct cause of CD and UC. The genetic aspects of CD have been linked by observing familial clustering of IBD cases[4]. Genetic variations in the *ATG16L*, *NOD2/CARD15* and *IL23R* genes have strongly been linked to the onset of CD[5]. Not only are individual gene mutations listed as a cause of CD, but a combination of them has also been shown in CD patients by conducting many population studies. There have also been many studies that have predicted surgical outcomes in both adults and children with specific genetic variations.

Diagnosis of CD can be tricky and requires a number of tests to be certain. Colonoscopy is the most effective way to diagnose CD but not in all cases because it only allows the physician to visualize the colon, ileum, and lower portion of the small intestine[6]. If the ulcers are located within the upper portion of the small intestine, this test will not be effective. In this case, a barium follow-through X-ray is useful because barium sulfate gives fluoroscopic images of the bowel and the physician can see areas of inflammation and narrowing[6]. Another effective method of diagnosis is the use of white blood cell scans. During this procedure, white blood cells are tagged with a radioisotope and then injected back into the patient. At specific intervals of time, the scan can locate accumulations of white blood cells in the intestine at the site of CD. This method is also useful to monitor the disease and show effectiveness in other therapies. Furthermore, a simple blood test can also diagnose CD because it can determine whether the patient is anemic or has a vitamin B12 deficiency because vitamin B12 is absorbed in the ileum and a deficiency can be due to ileitis.

CD causes a wide variety of symptoms and can be confused with UC, which is a similar disease, both under the group of IBDs. UC is only found in the colon and affects the mucosal membrane, whereas CD can occur anywhere throughout the gastrointestinal (GI) tract and affects the thickness of the GI wall. Individuals with CD can experience flare-ups followed by remissions. It can vary from one flare-up in a lifetime to multiple flare-ups that need surgical treatment. Symptoms include persistent diarrhea, abdominal pain in the affected area, fever, and weight loss[6]. There are also signs and symptoms that may occur unrelated to the GI tract, such as reddening and inflammation of the eye, joint pain, skin lesions, and sores inside the mouth.

Currently, there is no cure for CD. Treatment is focused on relieving the symptoms and putting it into remission. Since CD is characterized as an autoimmune disease, medications to suppress the immune system include 5-aminosalicylic acid and steroids, such as prednisone[6]. Antibiotics such as clarithromycin, ampicillin and metronidazole, can also be used. More than 50% of patients with CD will have to undergo surgical treatment to correct a fistula, drain an abscess, open a narrow or obstructed bowel, or remove a segment of infected intestine.

AUTOPHAGY-RELATED 16-LIKE 1 PROTEIN COMPLEX

Autophagy is a catabolic process of intracellular degradation in which cytoplasmic components are sequestered within vesicles and delivered to the lysosomes. Cells use this pathway during nutrient starvation because they can break down non-vital components and use them as nutrients. Autophagy plays a role during infection by helping rid the cell of foreign antigens by breakdown of the pathogen. This pathway can also be implemented as a repair mechanism to degrade damaged organelles and proteins. Autophagosomes, formed by the fusion of lysosomes and vesicles, are also implicated in the processing of intracellular bacteria. If the gene responsible for autophagy is mutated, it can cause a shift in normal flora as previously mentioned, and lead to many GI problems, which has been pointed out as a possible cause of CD. If the cell cannot regain nutrients or fight off foreign antigens within the GI tract, these cells will undergo programmed cell death and cause tissue damage. This damage can be seen as lesions and ulcers within the intestines, creating dead infected patches of tissue along the GI tract. The only option of treatment is surgery to remove the sections of the intestines with the diseased tissue so that the necrosis will not spread among neighboring cells.

An autophagosome, a double-membraned vesicle formed by autophagy, envelops part of the cytoplasm and delivers it to the lysosomes where it is degraded and recycled. There have been approximately 30 autophagy-related (Atg) genes identified, with two proteins having ubiquitin-like characteristics, Atg12 and Atg8[7]. These proteins covalently modify their target protein with molecules such as ubiquitin-like proteins to tag them for degradation. Both proteins also contain a conserved ubiquitin-fold region[7]. Autophagosomes use two conjugation systems, the Atg12 and LC3-Ⅱ systems[8]. These systems were first discovered during yeast genetic studies revealing a set of 17 ATG genes involved in the autophagy pathway[9]. In the Atg12 conjugation system, an Atg12-Atg5-Atg16L complex forms, and dissociates from the membrane just before or after completion of the autophagosome[8]. The ATG16L1 protein is expressed in the colon, small intestine, intestinal epithelial cells, leukocytes, and spleen[8]. Recent independent studies have shown that an ATG16L mutation, located on chromosome 2, is associated with the onset of ileal CD, and is therefore a key molecule in elucidating the genetic aspects of this disease[10].

Multiple studies have been performed with each resulting in the same conclusion that ATG16L is implicated in CD. During a genome-wide survey of 19 779 non-synonymous single nucleotide polymorphisms (SNPs), Thr300Ala within the N terminus of ATG16L was found to be highly associated with CD by using a haplotype and regression analysis[4]. This study used a total of 735 CD patients, 368 controls and 72 SNPs. A second report from the North American CD genome-wide study submitted by Rioux and colleagues also shows an association with ATG16L using a case-control analysis in 988 CD patients and 1007 controls[11]. Another group of German and British collaborators demonstrated that rs2241880, another non-synonymous variant of the *ATG16L* gene on chromosome 2q37.1, is implicated in the autophagy pathway[8].

In a study performed on an Italian cohort, the same polymorphism, rs2241880, was observed in 667 CD and 668 UC patients[12]. Both the frequency of the G allele and number of carriers of the G allele were increased in CD patients when compared to the controls[12]. These differences were only significant in the adult subgroup, which could be due to the small sample size of the pediatric subgroup. In comparison, there were no significant allele or genotype frequencies found between UC patients and controls for the groups as a whole[12]. During analysis of genotype/phenotype correlation of the rs2241880 SNP, there were no associations with disease location, behavior, and age at diagnosis based on the Montreal Classification of CD[12]. There were also no associations found in sex, smoking, and perianal fistulae[12]. For the rs2241880 variant, recent studies, specifically by Prescott[13], demonstrate an association with the ileal form of CD with or without colonic involvement, but not with isolated colonic disease[12].

In addition, a study from Oxford compared 645 CD patients with 1190 controls and showed an association of ATG16L1 with CD[14]. To understand fully the function of this gene, a study utilized oligo-based silencing RNA directed against ATG16L1 isoforms, where autophagy was induced by *Salmonella typhimurium* in ATG16L1 knockdown HEK293 cells. There was a significant difference between the knockdown cells com-pared to the control cells during the autophagy pathway[11]. It is clear to say that variants of this gene have been proven without a doubt to be directly associated with CD because autophagy plays a critical role in disease pathogenesis. Further research needs to focus on understanding how ATG16L1 variants contribute to disease susceptibility in IBD patients, and their possible therapeutic implications.

TUMOR NECROSIS FACTOR SUPER FAMILY 15

Tumor necrosis factor super family 15 (TNFSF15) is a Th-1 polarizing cytokine involved in systemic inflammation. TNF functions to regulate immune cells, induce apoptosis, induce inflammation, and inhibit tumorigenesis. TNFs are produced by macrophages, lymphoid cells, mast cells and endothelial cells. During immunological studies, it has been found that CD patients have an increased expression of TNFSF by multiple cells in the intestinal tissues when compared to controls[7]. The *TNFSF15* gene is a candidate for increased susceptibility in IBD. It binds to a specific T-cell receptor to enhance

cytokine-induced interferon expression in mucosal $CD4^+$ T cells[7].

In 2005, the first genome study involving IBD tested nearly 80 000 SNPs in Japanese CD patients. This study identified haplotypes within the *TNFSF15* gene, which included seven SNPs within a 280-kb region on chromosome 9q32[15]. By resequencing TNFSF15 from the same CD cases but with a new control group, TNFSF15 was found to be strongly associated with CD with an odds ratio (OR) of 2.17 (95% CI: 1.78-2.66), $P = 1.71 \times 10^{-14}$[15]. The Japanese wanted to see if the same patterns were seen in other population groups so they replicated their associate in two panels from Oxford, United Kingdom. Although the risk haplotype was identified in both cohorts, there was a weaker effect size ($P = 0.02$ in both family-based and case-control association panels). In addition, another study involving a Jewish cohort also showed an association of TNFSF15 with CD, and also suggested that in response to FC-gamma receptor stimulation, *TNFSF15* gene variation aggravates induction of TNFSF15[16]. However, in a separate study using a Belgian CD cohort, no significant association was observed between CD and TNFSF15[4]. This may have been due to different marker genotypes or differences in susceptibility genes between Asian and European cohorts[4]. Not enough studies have been performed regarding TNFSF15 and its possible implications in CD. Future studies have to focus on different populations to provide efficient insight.

NOD2/CARD15 GENE

Nucleotide-binding oligomerization domain containing 2 (NOD2), located on chromosome 16q12, is a protein that plays an essential role in the immune system by controlling commensal bacterial flora in the intestine[17]. NOD2 belongs to a nucleotide-binding domain, leucine-rich repeat family of cytoplasmic proteins that may detect a variety of bacteria by acting as an intracellular sensor for bacterial peptidoglycan[18]. It has the ability to respond to N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) *via* the leucine-rich repeat domain. The MDP is conserved in both Gram-negative and positive bacteria[10,19]. This leucine-rich repeat domain plays a role in protein-protein interactions and the middle portion of the protein is responsible for self-oligomerization. The N-terminal portion of NOD2 contains two caspase recruitment domains (CARDs) which play a role in apoptosis. The *CARD15* gene, which encodes for the CARD domain within NOD2, has been specifically identified as a genetic factor for CD. Three SNPs were found to be independently associated with CD: rs2066844, rs2066845, and an insertion mutation 3020insC[20]. Each variant may result in distinct phenotypic expression of CD[4]. To activate NOD2, Rip2 kinase is required because it is necessary for downstream signaling of NOD2 and signaling cascades such as nuclear factor (NF)-κB and mitogen-activated protein kinase cascades[21].

The intestinal mucosa is constantly exposed to a large number of commensal microorganisms; the majority of which inhabit the large intestine. In a healthy individual, there is a basal immune response elicited from the interaction between the intestinal immune system and commensal bacteria. This immune response is constantly present to protect the host from pathogenic and non-pathogenic bacteria. When there are changes within this balance, the intestines are susceptible to chronic intestinal inflammatory conditions, such as CD[21]. Multiple genetic studies have linked NOD2 with susceptibility to CD. However, NOD2-deficient mice do not develop colitis, suggesting that dysregulation of the NOD2 pathway is not sufficient to provoke CD[22]. This is not a surprise because the pathogenesis of CD is caused by several factors including environmental, dysfunctional immune system, and a shift in normal bacterial flora[21].

NOD2 is expressed in Paneth cells, which are found in the intestines. The exact functions of Paneth cells are still unknown, but they are likely to contribute to the host defense by secreting antibacterial compounds due to the presence of lysozyme. A NOD2 mutation can alter the function of Paneth cells, which alters their antimicrobial activity and leads to the development of ileal lesions, which correspond to the location of these cells. Of the three NOD2 mutations associated with CD, both rs2066844 and rs2066845 are the result of a two-amino-acid substitution in which rs2066844 is encoded by exon 4 and rs2066845 is encoded by exon 8[23]. The variant 1007fs is created by a frameshift mutation in exon 11[23]. Each of the three mutations occurs within or near the leucine-rich repeat and decrease the cells ability to activate NF-κB in response to peptidoglycan[18]. Interestingly, these mutations are observed in Caucasian patients but not in Japanese, Chinese and Korean patients with IBD, and they are very rare in African Americans with IBD[24,25]. An individual heterozygous for at least one NOD2 mutation is at a 2-4-fold increased risk of developing CD, whereas a homozygous individual for at least one NOD2 mutation is at a 20-40-fold increased risk when compared to healthy individuals[14].

Studies of each individual mutation in NOD2 have shown that 1007fs causes a decrease in defensin expression[26]. Defensins are cells of the immune system that assist in killing phagocytized bacteria. They function by binding to the microbial cell membrane and forming a pore in the membrane that allows outward flow of nutrients and essential ions. CD patients who are homozygous and/or heterozygous for NOD2 mutations typically have lower defensin levels in their ileostomy fluid[17]. As a result of a mutation in NOD2, the function of this protein is diminished, therefore allowing subsequent entry of bacteria into epithelial cells because they are no longer able to recognize them. This in turn alters the bacterial population in the intestines and thus, defensins are not able to function correctly because they are working in an impaired bactericidal capacity[22].

In a recent study by Van Limbergen *et al*[17], it was

proven that NOD2 is required for the regulation of commensal microbiota in the intestine. The regulation of NOD2 depends on the downstream kinase Rip2 because Rip2-deficient mice fail to establish and regulate commensal bacteria in their terminal ileum. NOD2-deficient mice do not develop spontaneous intestinal inflammation. In conclusion, the NOD2–Rip2 pathway is critical for the regulation of homeostasis between the body's normal bacterial flora and innate immunity[17]. As a controlled balance, it has been found that the expression of NOD2 and Rip2 is dependent upon and regulated by the presence of commensal bacteria[17]. This creates a negative feedback by which the commensal bacteria positively regulate NOD2, and in turn negatively regulate the normal flora. NOD2 mutations directly affect the ileum in CD patients, thus, it is possible that they are responsible for the composition of the bacterial flora in the terminal ileum[17]. This may facilitate both disease pathology and progression.

Although the exact mechanism by which NOD2 contributes to the control of commensals in the intestines is still not known, there are many possible theories proposed by this study. The first theory entertains the possibility that NOD2 regulates the commensal flora through the bactericidal activity of ileal crypt secretions[17]. The second theory is that NOD2 regulates the adaptive immune system by inducing lymphoid tissue genesis[17]. The third theory describes how NOD2 expression in myeloid lineage cells contributes indirectly to maintain the normal microbiota flora[17]. The fourth theory states that there may be other cells in the intestines, in addition to Paneth cells, that may play a role in the regulation of commensal bacteria in the intestines[17]. Further research is needed to elucidate this mechanism.

It has been reported that both adults and pediatric cohorts share a strong association between the NOD2 variants and ileal disease location[23]. Some studies have suggested that adult patients show a correlation in fibrostenotic behavior and NOD2 mutation status, whereas other studies have failed to replicate the same phenotypic effect[12]. It is necessary to study different CD cohorts from different countries to obtain an accurate effect of each mutation. Exploring the genotype-phenotype interactions in children is beneficial because they show a higher gene dosage and have less environmental influences.

A study by Lacher and colleagues has explored the association of the NOD2 mutation in German pediatric CD patients and the risk of surgery. The risk of surgery in children is important to predict because surgery is sometimes the only method of treatment. Out of 171 young CD patients, 78 (45.6%) carried at least one NOD2 mutation, with 11 being compound heterozygous, and 14 being homozygous for two NOD2 mutations[14]. The presence of rs2066844 was found in 29 (17%) children, rs2066845 was found in 18 (10.5%), and 1007fs was found in 42 (24.6%)[14]. Overall, one out of three German children with CD had at least one NOD2 mutation. In comparison, 36% of adult CD patients were heterozygous for at least one NOD2 mutation: 17.2% for rs2066844, 9.6% for rs2066845, and 11.7% for 1007fs[14]. In conclusion, the genetic alterations were observed more predominantly in German pediatric CD patients than in adults. The study also looked into each mutation and its association with localization and symptoms of the disease. A 4.73-fold increased risk of isolated ileal localization was observed in patients that were identified as 1007fs carriers when compared to children with none of the NOD2 mutations[14]. Although the 1007fs carriers were predisposed to ileal disease, there was no involvement of the ileocolonic or upper GI tract[14]. The next characterization the study explored was the association of NOD2 mutations and stricturing disease or perianal fistulae in their German pediatric CD cohort. Only 17% of patients showed a stricturing phenotype and 18.1% had a perianal fistula[14]. Among the children that showed a stricturing phenotype, 79.3% required surgery and those with the 1007fs mutation had surgical complications[14]. The outcome showed a 9.8-fold increase in surgical complications when the child carried at least one allele for the 1007fs mutation[14]. Not only is the 1007fs mutation strongly associated with isolated ileal disease, but children are at a high risk for surgical intervention. Therefore, this mutation can act as a prognostic tool in Caucasian children with CD[14].

Another study by Jurgens and colleagues has investigated the presence of fistulas and their association with NOD2 homozygosity and how they predict intestinal stenosis in CD patients. It was observed that patients with fistulas had simultaneous intestinal stenosis[15]. In another study using the same research, it was found that NOD2/CARD15 variants, especially with 1007fs homozygosity, could predict the occurrence of intestinal stenosis[27]. By using a strict screening process based on phenotypes including stenoses (stricturing CD) and fistulae (penetrating CD), and genotype, the study isolated a total of 145 patients. One hundred and twenty-five of the patients had penetrating CD with simultaneous stenosis within a 6-mo interval[28]. It was also found that all 14 CD patients homozygous for NOD2 variants suffered from stenosis[28]. To no one's surprise, 11 out of the 14 patients with stenosis and fistulas carried the NOD2 1007fs mutation[28]. These data confirm the strong risk factor of the 1007fs NOD2 mutation in the prevalence of intestinal stenosis, which may be related to the decreased intestinal barrier function found in CD patients with NOD2/CARD15 variant mutations[28]. This study has isolated the 1007fs variant as the cause in disrupting the intestinal barrier and causing the many problems often observed in CD patients.

To investigate whether stenosis occurs in a specific anatomical region, 223 patients with 248 stenoses located at different intestinal regions were examined[28]. The most common anatomical region was the terminal ileum with 68.8% of stenoses, followed by 11.7% found in the rectosigmoidal segment, and 9.3% in the jejunum or proximal ileum[28]. In conclusion, homozygosity in the NOD2/

CARD15 mutation is a strong risk factor for intestinal stenosis[28]. When the study looked into the possible association between stenosis and interleukin-23 receptor (IL-23R mutation variants, there was a weak connection with no influence on stenosis[28]. This study suggests classifying CD into four disease phenotypes instead of the current three in the Montreal Classification System. The four new classifications would be: non-stricturing[12], non-fistulizing CD[12]; stricturing, non-fistulizing CD[18]; non-stricturing, fistulizing CD[29]; and stricturing, fistulizing CD[28]. It is important to link any possible association between fistulas and stenoses because there is a strong risk factor for recurrence of CD after surgery[28].

Many studies have investigated North American, European and Asian countries and their CD patients with specific genotypes. Interestingly, the NOD2/CARD15 mutations are very rare and even absent in Asians (Japanese, Chinese and Korean), Arabs, Africans and African Americans[28]. A study by Baptista and colleagues has concentrated on a South American population for the first time. There were a total of 187 CD patients used for the study with a median age of 33 years and a median age of onset of 23 years[24]. Their patients were ethnically classified into the following groups: 58.8% were in the Brazilian subgroup, 36.9% shared a common European ancestry and were in the European-Brazilian subgroup, three were Amerindian-Brazilian, and two were Afro-Brazilian[24]. The alleles related to CD within the CARD15, rs2066844 and 3020insC variants were significant for CD susceptibility[24]. Both the rs2066845 and 1007fs variants failed to show any significant association[24]. Among their patients, 30% had at least one NOD2/CARD15 variant allele[24]. The frequency of rs2066844 (9.63%) in the Brazilian CD cohort was consistent with the reports for European populations[24]. In conclusion, this study confirmed that CARD15 variants lead to greater susceptibility to CD in the Brazilian population[24].

INTERLEUKIN-23 RECEPTOR

IL-23R is a protein consisting of an IL-12β1 and an IL-23R chain[27]. The molecular location of the *IL23R* gene is on chromosome 1 and is formed by the binding of IL-12p40 and a p19 protein[30]. It is highly expressed on the cell membrane of memory T cells and other immune cells, such as natural killer cells, monocytes, and dendritic cells, which identify foreign substances to defend the body against infection. It is highly involved in the mediation of proinflammatory activities by the production of IL-17 *via* the activation of Th17 lymphocytes[20]. IL-23R interacts with IL-23, which is a cytokine that regulates the activity of immune cells and plays an important role in the inflammatory response against infection by bacteria and viruses. It also has been suggested that the functional IL23R pathway polymorphisms play a role in modulating neonatal development of intestinal tolerance and bacterial colonization[4].

Th-17 lymphocytes are a distinct subset of T-helper cells, which mainly produce IL-17 and to a lesser extent IL-6 and TNF-α[26]. IL-17 *in vitro* and *in vivo* acts as a potent inflammatory cytokine and is involved in the destruction of cartilage and bone, as seen in rheumatoid arthritis[12]. It has been reported that IL-23 could be a key regulator in the differentiation of Th-17 lymphocytes from memory T cells. It also has been suggested that IL-23 plays a role in providing a survival advantage to already differentiated Th-17 cells[21]. The expression of the heterodimeric receptor complex, IL-23R and IL-12Rβ1, regulates activities of IL-23[12]. Therefore, the IL-23-IL17 cytokine axis is a key pathogenic mechanism that mediates the development and progress of inflammation by Th-17 cells. The role for the IL23-IL17 axis in CD patients was supported in human patients and animal models of colitis[31]. Both cytokines are increased in knockout mouse models of IBD. More specifically, IL-17 levels are increased in both intestinal mucosa and in the serum of CD patients[12]. In a study using IL-17R knockout mouse models, an association was found with colitis and disease severity[27]. Therefore, the use of anti-IL-12p40 antibody to treat CD patients is a therapeutic option because it is known to reduce production of IL-23 and IL-17 in the lamina propria cells[12]. The mechanism of IL-23R is clearly important to understand because it is directly associated with CD.

During a genome-wide association study, 2877 DNA samples from IBD patients (two-thirds CD and one-third UC), identified rs11209026 as a possible protective variant, in the *IL23* gene on chromosome 1p31[4]. There are many other variants within IL23R that are associated with IBD, but rs11209026 has the strongest association with conferring protection against CD[4]. Although the effect of IL23R variants is greatest in CD, it may have an overall effect on susceptibility to chronic intestinal inflammation.

Additional studies have confirmed the susceptibility of the *IL23R* gene to CD in North American and European populations. They include cohorts ranging from Scottish pediatric IBD, Belgian CD and an independent cohort of 883 families[4]. Due to the role of IL-23 in activation of inflammatory responses, targeting this pathway may be a good therapeutic approach. Some promising research is underway using anti-p40, which blocks IL-23 and IL-12 activities. The variant allele, rs11209026, could be exploited to define clinical outcomes, such as a pharmacological approach to mimic the rs11209026 polymorphism[4].

A study by Schmechel and colleagues has shown a link in susceptibility to CD with Th17 cell function, IL-22 serum levels, and IL23R genotype. IL-22 is a strong activator of proinflammatory gene expression and upregulates SOC3 mRNA in intestinal epithelial cells[29]. Recent evidence has shown that Th17 cells expressing IL23R play a key role in the mechanism by which IL23R modulates IBD susceptibility[32]. Previous studies have shown that Th17 plays a role in autoimmune diseases,

such as rheumatoid arthritis and CD. However, Th17 is responsible for the important function of antimicrobial immunity at epithelial barriers where it produces cytokines such as IL-22. Because it produces IL-22 in epithelial barriers such as in the intestines, theoretically by testing for increased IL-22 serum levels, a physician can determine CD and disease activity.

It has been confirmed that IL-22 serum level is increased in CD and correlates with disease activity[33]. IL-22 serum levels are also independent of CD phenotype and CARD15 genotype, but are modulated by IL23R polymorphisms[33]. The study investigated IL-17 serum because Th17 cells also produce IL-17. There was no correlation between IL-22 and IL-17 serum levels[33]. Currently, serum levels of TNF-α and IL-6 are used as inflammation markers in determining CD. There was no difference in TNF-α and IL-6 levels whether CD was active or in remission, whereas IL-22 levels were significantly higher in active CD compared to CD in remission[33]. Therefore, measuring IL-22 levels are clinically relevant in determining the disease activity in CD patients[33].

There are also strong associations between IL-22 serum levels and *IL23R* gene variants. Previous studies have shown both protective and inducing variants of IL23R in susceptibility to CD. Although rs1004819 is the CD increasing variant of IL23R, rs11209026 has shown to be a protective IL23R variant against CD. As predicted, when the IL-22 mean serum levels were tested against each variant, the serum levels were highest among SNPs that increased CD risk as opposed to the identified protective SNPs of IL23R, which had low serum levels[33]. In contrast, the three main variants within NOD2/CARD15 did not show any differences in IL-22 serum levels[33]. Although, the exact function of IL-22 in human IBD is still unknown, recent studies have observed increased β-defensin-2 expression and intestinal epithelial cell migration and proliferation upon stimulation with IL-22[29]. These data ultimately suggests a clinically useful marker to assess disease severity and Th17 cell activity in CD patients[33].

As mentioned above, a rare glutamine allele, rs11209026, in IL23R conferred protection against CD during a recent genome-wide association study. Recent studies have proven that this variant protects against CD in both Jewish and non-Jewish populations. In addition, in a study involving IBD (both UC and CD) patients of Spanish Caucasian origin, the rs11209026 variant was seen to be most significantly associated with IBD protection (OR: 0.4; 95% CI: 0.3-0.7)[34]. A study by Dubinsky and colleagues has investigated this rare allele further and how it protects pediatric CD patients. They used the transmission disequilibrium test (TDT) analysis and genotyping of whole blood samples from children with IBD and their two parents. This rare rs11209026 SNP was present in 2.67% non-Jewish CD patients and 2.94% of non-Jewish UC patients[35]. The TDT demonstrated that the allele was under-transmitted in all CD offspring and confirmed the negative association between rs11209026 and CD[35].

Another way to confirm the association of alleles with CD and UC is to use a population that is genetically isolated, such as the Finnish population. This will provide an advantage in molecular genetic studies in complex disorders[36]. A study by Lappalainen has used such a strategy to confirm the association of IL23R, TNFRSF1A, and the HLA-DRB1*0103 allele variants. The strongest association of IL23R with the marker rs2201841 (IL-23R risk variant) showed a frequency of 37.2% in CD. In most studies, the non-synonymous SNP, rs11209026 (the protective IL23R allele), has had the highest association, but is only marginally associated with Finnish CD[36]. No association has been observed between the IL23R markers and UC patients[36].

Previous studies have shown that the HLA-DRB1*0103 allele is associated with both UC and CD. In the Finnish population, this allele only has a frequency of 0.6%, which is not statistically significant, whereas it is significant for UC and IBD[36]. When looking into the genotype-phenotype association, patients carrying the rare HLA-DRB1*0103 allele have colonic involvement in CD[36]. During the TNFRSF1A analysis, the investigators genotyped a rare A36G variant and an IVS6+10A (rs1800693) variant. CD patients with both variants often show ileocolonic disease in comparison with patients without these two variants[36]. The weak association is probably due to the small sample size. Interestingly, when the protective haplotype of IL-23R (described in the above paragraph) was sequenced and compared to North American Caucasian CD patients, there was a one-nucleotide difference between Finnish (CCTGATCG) and North American (CGTGATCG) CD patients[36]. In conclusion, the HLA-DRB1*0103 allele has been confirmed in CD patients, which shows an inherited susceptibility of colonic inflammation. The *TNFRSF1A* gene variants are markers of ileocolonic involvement in CD[36]. Although this study shows a weaker association of the *IL23R* gene, it confirms the genetic involvement within a Finnish population.

In another population study among French-Canadian and English-Canadian children, the association between genetic variants of the *IL23R* gene and early-onset of CD has been investigated. To study the associations accurately, they have carried out both a case-control and a family-based study. They have targeted the 10 SNPs in IL23R achieved by the genome-wide study and the three CARD15 SNPs. In total, 259 CD patients and 139 controls were recruited with a mean age at diagnosis of 13.3 years (range: 2.6-20 years)[12]. The IL23R protective allele, rs11209026, was only present in 2% of CD case chromosomes and 6% of the control chromosomes[12]. All CARD15 variant allele frequencies were higher among CD patients when compared to the control group[12]. In the *IL23R* gene, they observed a significant association among four SNPs that did not posses any CARD15 variants[12]. Therefore, variants in the *IL23R* gene were associated with early-onset CD among Canadian children.

This study confirms previously reported findings in CD patients among North Americans.

In continuation of a study that was described in the ATG16L section of this paper, Latiano and colleagues have shown that the replication of IL23R is associated in adult and pediatric onset of IBD in Italy. Approximately 730 CD patients were genotyped for the rs7517847 and rs11209026 variants. For the rs7517847 polymorphism, significant reductions were found in minor allele (G) frequency in CD patients when compared with controls and in the number of carriers[12]. No differences were found in UC patients. When the rs11209026 polymorphism was examined, a significant increase in the frequency of the risk genotype was observed in CD patients. In either SNP, there were no correlations between phenotypes of CD and risk alleles or genotypes of the *IL23R* gene.

To specify which IL23R variant is the main disease associated variant, a study using German CD patients was conducted. Among all 10 IL23R SNPs, they chose to focus their study on the rs1004819 variant because it had the strongest association to CD when compared to controls. This variant showed high prevalence of ileal involvement when carriers had the TT genotype versus the CC wild-type genotype[28]. This identification is different from recent data published by Roberts *et al*[37], which have identified the rs7517847 variant as having the strongest association with CD, along with other overlapping North American study populations. They have only analyzed ileal cases of CD, therefore, it is assumed that IL23R variants are predisposed to an ileal disease phenotype[37]. In a recent British study, they could not identify any association between disease phenotype and IL23R variants[9]. Interestingly, they found that the rs1004819 variant was 1000-fold weaker than that reported in the German study.

Not only are the IL23R variants highly associated with CD, they are also associated with other chronic inflammatory diseases, such as the rs10489629 variant with chronic periodontitis. In psoriasis, the variant rs11209026 has been described as a predisposing haplotype[17]. In both CD and psoriasis, treatment with an anti-p40 IL-12/23 antibody has shown promising results. Antibodies to the p40 subunit block both IL-12 and IL-23, although in knockout mice studies, it has been proven that IL-23 drives chronic intestinal inflammation[28]. Therefore, IL23R can serve as a therapeutic target in many different chronic inflammatory diseases, although the variants may differ among the different diseases. It is hypothesized that this is due to alternative mRNA splicing, which results in corresponding IL23R isoforms with different tissue distribution[28].

IBD5 GENE

The *IBD5* gene is about 250 kb and is located at position 5q31. During a genome-wide linkage analysis, mapping studies have identified a risk haplotype within the IBD5 locus. Many studies have linked this gene and its two variants, SLC22A4 (OCTN1) and SLC22A5 (OCTN2), to CD. IBD is believed to originate from an uncontrolled mucosal immunity of the GI tract[17]. Although these variants are associated with CD, they act independently because there is no statistical evidence for interaction between *IBD5* and the *IL23R*, *ATG16L1* or *CARD15* genes[12].

CTLA4 VARIANTS

The *CTLA4* gene is a member of the immunoglobulin superfamily and is expressed on the surface of helper T cells. It is located within the 2q33 region, translates into a protein that plays a role in the immune system, and may have a genetic association with IBD. It is a T-cell suppressor, which is essential in the function of the $CD25^+$ $CD4^+$ regulatory cells[7]. These regulator cells control the process of intestinal inflammation. Many SNPs have been studied within this gene and it has been found that the rs3087243 variant shows the most association with IBD followed by the rs11571302, rs7565213 and rs11571297 variants[35]. Although studies have shown that the three variants in the *CTLA4* gene, g.49A > G (rs231775), g.-318C > T (rs5742909), and rs3087243, have no association with CD, other work has suggested that these variants may control the phenotype of CD[14].

A study performed by Hradsky has shown no crude association between CD and SNPs within the CTLA4 gene[12]. The study explored the possibility of interactions in CTLA4 SNPs with variants in IL23R and NOD2. The R-project package SNPassoc was used and significant interactions between the three CTLA4 variants with NOD2 p.Leu1007fsX1008 and IL23R rs11209026 were observed[12]. This may be due to complex gene-gene interactions. To characterize further the different variants and whether they determine phenotype in CD patients, the study used a case-only design. They observed a difference of minor allele frequency at the rs3087243 gene between pediatric-onset and adult-onset of CD[12]. It seems that a genetic factor has a greater impact in early-onset patients when compared to the adult-onset patients[38]. Within this study, the age of diagnosis and localization of the disease was strongly associated with the rs3087243, rs11571302 and rs11571297 variants[12].

NOD2, IL23R, OCTN1/2 AND ATG16L1 POLYMORPHISMS

Gene-gene interactions

Interactions of the major IBD alleles show a high susceptibility in CD patients. Gene-gene interaction can either enhance or weaken the effects of an individual gene, therefore making it more important than independent studies looking into the effects of single susceptibility genes. Csongei and colleagues have performed a gene-gene interaction analysis in the Hungarian CD population[5]. They concentrated on the two IL23R gene risk variants (rs2201841 and rs1004819), the ATG16L1 gene variant (Thr300Ala), and the three NOD2/CARD15

variants (rs2066844, L1007fs and rs2066845). Logistic regression analysis showed that the IL23R variants, both rs1004819 and rs2201841 and the NOD2/CARD15 variants (rs2066844 and L1007fs) conferred significant risk for CD. When the patients were homozygous for IL23R (rs1004819 and rs2201841) variants or ATG16L1, there was a highly increased risk for CD. When they analyzed possible statistical interactions between pairs of ATG16L1, IL23R and CARD15 variants, no evidence of interactions was found. Therefore, all examined loci contribute independently to CD risk. Although significant statistical interactions were not detected, these susceptibility factors may have a cumulative effect in the Hungarian population.

Further gene-gene interaction studies have been performed in another population study, as well as assessment of CD genetic risk factors. The study included five SNP variants for IL23R, the functional variants SLC22A4 and SLC22A5, and the ATG16L1 mis-sense risk polymorphism Thr300Ala. All the SNPs, except for rs1495965 of IL23R, showed significant association when carriers were either homozygous or heterozygous for the alleles[2]. Among them all, rs10889677 of IL23R, showed the strongest association with CD risk[2]. Unsurprisingly, ATG16L1 also showed a strong association with CD[2]. None of the SNPs showed an association with UC. When they evaluated correlations between genotype and phenotype for intestinal complications, carriers of the rs7517848 allele of IL23R, particularly those who were homozygous for the allele, were found to be at risk for ileal disease. No other SNPs showed differences in genotype or carrier frequencies when compared to CD patients and their history of complications.

Homozygote variants from the IBD5 and ATG16L1 genes had a greater risk than heterozygotes, suggesting a gene dosage effect[2]. When SNPs were considered two at a time, the best interactions were shown between IL23R_rs10889677 and IBD5_rs17622208, which were not statistically significant[2]. However, when using a logistic regression approach between these two markers, the IBD5_rs17622208 risk was only significant in the presence of the IL23R_rs10889677 risk allele[2]. When SNPs were considered three at a time, the model suggested statistically significant interactions between IL23R_rs10889677, IBD5_rs11739135, and ATG16L1_rs2241880[2]. When SNPs were considered four at a time, the model suggested interaction between IL23R_rs2201841, IL23R_rs7517847, and IBD5_rs11739135[2]. A study in Oxford, UK has hown that certain IL23R polymorphisms have an association with CD only when the person is positive for IBD5[2]. In conclusion, the logistic regression model did not show any significant evidence of gene-gene interaction, due to the small size of the study, even though there was a consistently small association between IBD5 and IL23R.

Childhood- vs adult-onset CD

The pathogenesis of pediatric and adult IBD differ. The age of childhood and adult onset varies between cultures to culture because different cultures base it on different aspects such as physical, mental, or puberty. North American studies choose an age cut-off of 18 years, and < 18 years is considered a child, even though an individual at this age is physically mature. This is why Canadian studies have chosen to describe a child as < 16 years old. The belief is that the lower the age cut-off, the better the results are when comparing pediatric and adult onset of disease. Recent studies have shown that a subgroup of patients with early-onset IBD may have specific phenotypes that differ from adult-onset IBD[39]. Many believe that pediatric-onset IBD is influenced by genetics compared to adult onset because there is less time for exposure to environmental modifiers to influence the onset of disease. Adult-onset is probably due to a mixture of genetics and abundant environmental exposure[25]. For example, smoking is a major variable in adult IBD patients, but has little influence on pediatric IBD cohorts. Currently, there are conflicting studies on whether NOD2/CARD15 polymorphisms are associated with the age of onset of IBD because some show an association towards a younger age, while others show no effect[40].

A study by Gazouli *et al*[25] has investigated the main polymorphisms and their association with childhood-onset of CD in a Greek cohort. While investigating the genotype and allele frequencies of the NOD2/CARD15 polymorphisms, rs2066844, rs2066845 and 3020insC, a statistically significant association between rs2066844 and adult-onset CD was observed. In both pediatric- and adult-onset CD, individuals with at least one NOD2/CARD15 polymorphism showed a genotype-phenotype correlation with ileal involvement[25]. The study also confirmed the recently described association between IL23R variants in both child- and adult-onset CD[35]. There has been conflicting evidence in studies regarding the ATG16L1 SNPs. One study has shown no association with early-onset or adult-onset CD[25]. Recent research has indicated that the ATG16L1 rs2144880 variant is associated with adult-pediatric-onset CD, whereas other studies have demonstrated an association with diagnosis at an earlier age[25]. In conclusion, the 3020insC variant in the NOD2/CARD15 gene is associated with CD and occurs considerably more often in childhood- than in adult-onset patients with CD[25].

There is growing evidence that pediatric-onset IBD shows distinct differences when compared to its adult counterpart. Familial aggregation studies have shown an age-adjusted risk of developing IBD in first-degree relatives of affected individuals compared to the general population[23]. The risk increases to > 30% for children when both parents are affected with IBD, suggesting that family history is the strongest risk factor[5]. This is especially true among CD patients. Familial cases of CD occur at a younger age with greater severity than random sporadic cases[36]. One main difference observed between pediatric- and adult-onset is that early onset shows a distinct and

more aggressive phenotype, such as the need for surgery, than similar IBD in individuals > 20 years old[41].

Although NOD2/CARD15 mutations are neither sufficient nor necessary for the development of IBD, they are associated with a younger age of onset, presence of ileal involvement, and the development of strictures[23]. There is also a gene dosage effect for CD location and complications. For example, stricture complications occur more frequently in CD children with the 1007fs mutation in the *NOD2/CARD15* gene compared to children without this variant due to early surgery[42]. Therefore, children with this mutation have a sixfold increased risk for developing a stricture complication[23].

There has been conflicting evidence among studies that have attempted to show a link between the IBD5 locus and early-onset CD. A study by Rioux has found that the IBD5 locus is associated with early-onset CD where children were defined with an age of onset of < 16 years old[23]. However, studies from pediatric-onset CD cohorts have demonstrated that the risk of IBD5 is lower compared to that in adult-onset CD, while others have demonstrated enhanced risk of developing CD when an individual has both SLC22A4-A5 and NOD2/CARD15 mutations[43]. This connection may be due to a common pathophysiological mechanism[23].

Although there is no sex difference among patients with adult-onset IBD, there is a clear and distinct difference among children with CD. Many studies from pediatric CD cohorts in the United States, Canada and the United Kingdom have shown an increase in male incidence. The higher male to female ratio continues to be unexplained. These differences are not observed among UC pediatric patients[23]. It seems that sex is an age-dependent variable that has more influence on children than adults with IBD.

There are also phenotypic differences, such as disease location, among children and adult CD. For example, increased rates of upper GI tract disease and pure colonic disease in pediatric-onset CD have been identified. This difference may be due to the amount of examination during the onset of disease. Children undergo extensive GI endoscopy, whereas adults do not[23]. These findings may be artificial or represent a true disease distinction among children and adults. Another distinction is the occurrence of colon-predominant disease during childhood-onset IBD under the age of 10 years old. With children < 5 years old, all have colon-only disease[44]. During a study of approximately 1400 North American early-onset patients, data showed a colon-predominant phenotype in children < 8 years old. In another study in Europe, the acquisition of ileal CD became increasingly common as an individual approached 16 years old[23]. These data confirm an association of colon-predominant phenotype in early diagnosed children that changes as they grow older.

Population studies with NOD2, IL23R and ATG16LI polymorphisms

NOD2, IL23R and ATG16L1 polymorphisms were studied in a Lithuanian cohort with IBD. The study included 57 unrelated patients with CD, 123 with UC and 186 healthy individuals as controls. The three NOD2 variants, the IL23R variant rs11209026, and the ATG16L1 variant Thr300Ala, were genotyped among the population sample. No individuals were carriers of all three NOD2 risk alleles, whereas two CD patients were compound heterozygotes[10]. Carriers of at least one NOD2 variant were highest among CD patients[10]. There were no significant differences observed between UC patients and the controls. The NOD2 variant, Leu1007insC, was significantly associated with increased susceptibility in the Lithuanian CD population[10]. In comparison to the other two NOD2 variants, the frequencies were very low and not significant in controls and among the IBD patients[10]. In contrast to other European studies, a positive association between rs2066844, rs2066845 and CD was not found[10]. When they further tried to analyze IL23R and ATG16L1, they were unable to replicate previous findings of increased susceptibility to IBD within the Lithuanian population[10]. They were was a trend for a possible association with the ATG16L1 risk allele[10]. It is of particular interest to study this population because Baltic countries have low IBD incidence rates, especially for CD. To confirm distinct IBD subtypes, a study using a larger North-Eastern European IBD sample needs to be investigated.

Another population study was performed among New Zealand Caucasians with IBD. Their cohort included 466 UC patients, 496 CD patients and 591 controls. All individuals were genotyped for the IL23R rs11209026 SNP, the ATG16L1 rs2241880 SNP, and the CARD15 variants. Significant interaction was detected between variants in ATG16L1, IL23R and CARD15 and CD susceptibility, whereas no significant association was observed between IL23R or ATG16L1 genotypes and IBD sub-phenotypes[37]. The strongest association occurred between the ATG16L1 rs2241880 variant and CD, with no association detected with UC[37]. ATG16L1 is suggested to have a CD-specific susceptibility locus. Unlike ATG16L1, the IL23R rs11209026 variant was strongly associated with both CD and UC. In their patient cohort, there was no evidence that IL23R or ATG16L1 genotypes influenced disease behavior, age of onset, location, or the need for surgical bowel resection[37]. On the other hand, CARD15 was consistently a susceptibility factor and predictor of CD phenotype. Similar to many other studies, all three CARD15 SNPs are significantly overrepresented in patients with IBD family history, early onset of disease, ileal disease involvement, and development of complications[33].

Interactions between NOD2 and IL23R variants with toll-like receptor-9 polymorphisms

Toll-like receptors (TLRs) are single, membrane-spanning proteins that play a key role in the innate immune system. These receptors recognize microbes that have structurally conserved molecules that breach the physical

Table 1 Key gene polymorphisms and their significance in Crohn's disease

Gene	Polymorphism	Relationship significance	Ref.
ATG16L	rs2241880	Associated with ileal form of CD with or without colonic involvement	[4,12]
	Thr300Ala	Highly associated with CD	
NOD2/CARD15	rs2066844, rs2066845, 3020insC, 1007fs	Independently associated with CD	[20]
IBD5	SLC22A4 (OCTN1), SLC22A5 (OCTN2)	Independently associated with CD	[12]
CTLA4	rs3087243, rs11571302, rs11571297, rs7565213	Associated with IBD; no crude association to CD	[35]
TNFSF15	80 000 SNPs tested, including 7 SNPs within a 280 kb region on chromosome 9q32	Strongly associated with CD for Japanese and Jewish cohorts, but not for Europeans	[4,15,16]
IL23R	rs11209026	Strongly associated with conferring protection against CD	[4,33]
	rs1004819	Highly associated with CD	

SNPs: Single-nucleotide polymorphism; CD: Crohn's disease.

barriers. Once bound, TLRs activate the immune system. The responsiveness of the GI tract to luminal bacteria is dependent on the interaction of transmembrane TLRs and the intracellular NOD2 receptor[18]. Specifically, TLR9 plays a role in the maintenance of intestinal inflammation in IBD. TLR9 is also responsible for stimulating NOD2, which in turn enhances innate immune responses. Patients with two NOD2 mutations lose the synergistic affect between NOD2 and TLR9 stimulation. Therefore, interactions of both receptors have implications for intestinal homeostasis and inflammation[18]. The TLR9 gene is located on chromosome 3p21.3, which is close to other CD susceptible loci[9]. There are four SNPs in TLR9, but two of them are sufficient to distinguish between the haplotypes, which are rs5743836 and rs352140.

There might be a synergistic effect of NOD2 and TLR9 stimulation, therefore, Torok and colleagues have tested for gene interactions between TLR9 and CD-associated variants of NOD2[18]. Significant associations between the two were observed that were specific to CD[18]. The controls and UC showed no difference in distribution of TLR9 polymorphisms and NOD2 variants[18]. Other CD variants in IL23R, ATG16L1 and IBD5 were analyzed for epistatic interactions. Aside from NOD2, the most significant association was found in the IL23R variant rs1004819, with ATG16L1 showing weaker associations with CD. There was no significant association between TLR9 polymorphisms with CD or UC phenotypes[18]. Along with previous studies, they also showed that NOD2 mutations were associated with younger age of diagnosis of CD, ileal disease, and need for surgery[18]. When there were two NOD2 mutations, there was a higher frequency of penetrating disease[18].

In conclusion, a new association between CD and a TLR9 polymorphism has been found. There is evidence that when CD patients carry CD-associated NOD2 variants, they have an increased incidence of TLR9 polymorphisms. This is not surprising because there is a synergistic effect of NOD2 and TLR9 stimulation and it is important for the maintenance of intestinal homeostasis and inflammation. TLR9 also demonstrates significant epistatic interactions with IL23R variants, but unlike NOD2, there is no association with the frequency in TLR9 polymorphisms present. This study shows the first evidence for interaction between polymorphisms in TLR9 and variants between NOD2 and IL23R[18].

CONCLUSION

CD is an autoimmune disease characteristic of chronic intestinal inflammation and lesions. It can affect people of all ages and ethnicities worldwide. Recent genome-wide studies have shown significant genetic associations of several variants and susceptibility to CD. This is true for North American, South American and European populations. Certain variants have been linked only to Asian and African cohorts. It is vital to understand the pathology of CD and the underlying genetic interactions to increase efficiency of diagnosis and develop drugs that target specific immune system pathways. There have been several genetic variants highly associated with CD, which include ATG16L1, TNFSF15, NOD2/CARD15, IL23R and IBD5 (Table 1).

The process of autophagy is an important aspect of our immune system. It is a way to destroy foreign pathogens that enter the body. The Thr300Ala variant within the N terminus of ATG16L, which is part of a complex that forms autophagosomes, has significant associations with CD. Different ethnicities show different genotype markers. This is seen in TNFSF15, which is involved in systemic inflammation and regulation of various immune cells. Seven haplotypes have been identified within a Japanese cohort, but not in other ethnic cohorts. More research needs to be conducted to characterize fully this possible genetic link to CD. The most significant association with CD is observed among NOD2/CARD15 and IL23R variants. NOD/CARD15 plays an essential role in maintaining the intestinal normal flora. There are many theories about the cause of CD, and one of them includes a shift in the intestinal bacterial flora. Therefore, it is no surprise that NOD2 variants are highly associated with CD. There are three independent variants associated with CD: rs2066844, rs2066845 and 1007fs. The other highly associated genetic link is observed within the IL23R gene. One variant that is highly associated with CD is rs1004819, whereas rs11209026 confers protection against CD. IL23R plays an essential role in

mediating proinflammatory activities. Another highly associated genetic link with CD is observed in two variants within IBD5. The two variants, SLC22A4 (OCTN1) and SLC22A5 (OCTN2), show significant association with CD, but no interactions between these variants and other CD-associated genetic variants have been observed. The last highly associated genetic link with CD is observed within the *CTLA4* gene. This gene plays a role in the immune system. The four polymorphisms associated with IBD are rs3087243, rs11571302, rs7565213 and rs11571297.

Many CD variants are associated with a specific CD phenotype, age of diagnosis, severity and location of CD, and surgical outcome. Gene-gene interactions have also been characterized among the different variants and other immune system receptors. We need to understand fully the pathogenesis of CD to target pathways for effective treatment. Further research is needed to explore all possible gene-gene interactions due to a gene dose affect associated with CD. It seems that new genetic associations are constantly being uncovered within IBD and then associated with either CD or UC. Overall, CD shows a greater genetic link than UC. Recent discoveries have led to therapies and treatments that show much promise. Researchers need to continue the search for genetic links and diseases because this may be the only way to understand the pathology of CD and develop effective treatments.

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ORIGINAL ARTICLE

Effect of *Helicobacter pylori cdrA* on interleukin-8 secretions and nuclear factor kappa B activation

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Abstract

AIM: To investigate genetic diversity of *Helicobacter pylori* (*H. pylori*) cell division-related gene A (*cdrA*) and its effect on the host response.

METHODS: Inactivation of *H. pylori cdrA*, which is involved in cell division and morphological elongation, has a role in chronic persistent infections. Genetic property of *H. pylori cdrA* was evaluated using polymerase chain reaction and sequencing in 128 (77 American and 51 Japanese) clinical isolates obtained from 48 and 51 patients, respectively. Enzyme-linked immunosorbent assay was performed to measure interleukin-8 (IL-8) secretion with gastric biopsy specimens obtained from American patients colonized with *cdrA*-positive or -negative strains and AGS cells co-cultured with wild-type HPK5 (*cdrA*-positive) or its derivative HPKT510 (*cdrA*-disruptant). Furthermore, the cytotoxin-associated gene A (*cagA*) status (translocation and phosphorylation) and kinetics of transcription factors [nuclear factor-kappa B (NF-κB) and inhibition kappa B] were investigated in AGS cells co-cultured with HPK5, HPKT510 and its derivative HPK5CA (*cagA*-disruptant) by western blotting analysis with immunoprecipitation.

RESULTS: Genetic diversity of the *H. pylori cdrA* gene demonstrated that the *cdrA* status segregated into two categories including four allele types, *cdrA*-positive (allele types; Ⅰ and Ⅱ) and *cdrA*-negative (allele types; Ⅲ and Ⅳ) categories, respectively. Almost all Japanese isolates were *cdrA*-positive (Ⅰ: 7.8% and Ⅱ: 90.2%), whereas 16.9% of American isolates were *cdrA*-positive (Ⅱ) and 83.1% were *cdrA*-negative (Ⅲ: 37.7% and Ⅳ: 45.5%), indicating extended diversity of *cdrA* in individual American isolates. Comparison of each isolate from different regions (antrum and corpus) in the stomach of 29 Americans revealed that *cdrA* status was identical in both isolates from different regions in 17 cases. However, 12 cases had a different *cdrA* allele and 6 of them exhibited a different *cdrA* category between two regions in the stomach. Furthermore, in 5 of the 6 cases possessing a different *cdrA* category, *cdrA*-negative isolate existed in the corpus, suggesting that *cdrA*-negative strain is more adaptable to colonization in the corpus. IL-8 secretions from AGS revealed that IL-8 levels induced by a *cdrA*-disrupted HPKT510 was significantly lower ($P < 0.01$) compared to wild-type HPK5: corresponding to 50%-60% of those of

wild-type HPK5. These data coincided with *in vivo* data that an average value of IL-8 in biopsy specimens from *cdrA*-positive and *cdrA*-negative groups was 215.6 and 135.9 pg/mL, respectively. Western blotting analysis documented that HPKT510 had no effect on CagA translocation and phosphorylation, however, nuclear accumulation of NF-κB was lower by HPKT510 compared to HPK5.

CONCLUSION: Colonization by a *cdrA*-negative or *cdrA*-dysfunctional strain resulted in decreased IL-8 production and repression of NF-κB, and hence, attenuate the host immunity leading to persistent infection.

Key words: *Helicobacter pylori* cell division-related gene A; Genetic diversity; Host immune response; Interleukin-8 secretion; Nuclear factor kappa B; Persistent infection

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative spiral bacterium, colonizes the human stomach and causes chronic inflammation, which may progress to peptic ulceration, atrophic gastritis and gastric cancer[1]. Although the *H. pylori* population structure appears to be clonal over short periods of time, isolates obtained from different individuals exhibit substantial genetic diversity, consistent with extensive recombination and a panmictic population structure[2-7]. Putative mechanisms for the generation of diversity within *H. pylori* include frequent horizontal genetic exchange among strains and a high level of spontaneous mutation occurring over a long evolutionary time period within a highly restricted niche[6,8].

Infection by cytotoxin-associated gene A (*cagA*)-positive *H. pylori* is a known risk factor for the development of gastroduodenal disease due to major changes in cellular morphology and the release of molecules, including cytokines, from the gastric epithelium. *H. pylori* infection up-regulates secretion of various inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor α, and contributes to the pronounced inflammatory response[9]. IL-8 production induced by *H. pylori in vitro* and *in vivo* is recognized as a host response to microbes[10,11], but the role of CagA on IL-8 production is less clear[12]. IL-8 is a potent pro-inflammatory cytokine which regulates neutrophil infiltration of gastric mucosa in *H. pylori* gastritis and is released through the signaling pathway concerning nuclear factor-kappa B (NF-κB), which includes extracellular signal-regulated kinase (ERK) activity[12,13] and mitogen-activated protein kinase[14]. Among the strongest transcriptional regulators of *H. pylori*-induced cytokine expression is the NF-κB family of transcription factors[15]. Activation of NF-κB can affect the expression of several hundred genes, and activation of its signal transduction pathway occurs in response to a wide range of stimuli and results in nuclear accumulation of NF-κB transcription factors, causing changes in the expression of target genes involved in innate and adaptive immunity, and inflammation.

We described previously that the *H. pylori* cell division-related gene A (*cdrA*) not only has a repressive role on cell division but is also involved in cell elongation and cell death *via* cell wall synthesis at the division site[16,17]. The *cdrA*-disrupted mutant HPKT510 was able to survive for the long-term in liquid medium, even under serum-free and aerobic conditions, and was more resistant to bactericidal of beta-lactam antibiotics than the wild-type HPK5[16]. Inactivation of *cdrA* during infection in the stomach may have contributed to ensuring persistent infection by altering its ability to adapt to the microenvironment. In fact, the *cdrA* gene was found to be absent in 3 out of 4 colonies recovered from a mouse infected with *H. pylori* strain B128[18]. Furthermore, additional isolates of the sequenced *H. pylori* strain J99 from a patient after a 6-year interval were subjected to microarray analysis, which indicated that the *cdrA* gene was missing in additional isolates[19]. Thus, we hypothesize that a loss of *cdrA* in *H. pylori* during infection might be an evolutionary event to alter its biological characteristics, which affects the host immune response to the microbes and promotes persistent infection.

In this study, the level of IL-8 secretion induced by a *cdrA*-negative strain was approximately 50% lower than those induced by a *cdrA*-positive strain *in vitro* and *in vivo*. Genetic diversity of *cdrA* was extended in American isolates and *cdrA*-negative isolates might be more adaptable to colonize in the corpus. Western blotting analysis documented that the CagA status at the point of its translocation and phosphorylation was not different between HPK5 and HPKT510 strains. However, expression of NF-κB was lower in HPKT510 than that in HPK5, indicating that *H. pylori* with inactivation of *cdrA* might escape from rigorous immune clearance and facilitate chronic persistent infection caused by decreased levels of IL-8 and nuclear accumulation of NF-κB.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Wild-type *H. pylori* HPK5 and its derivatives such as HPKT510, a *cdrA*-disrupted mutant carrying *xylE-Km* cassette[17], and HPK5CA, a *cagA*-disrupted mutant carrying *xylE-Km* cassette[20], were cultivated in Brucella broth (Becton Dickinson, United States) supplemented with 10% horse serum at 37 ℃ in an atmosphere containing 10% CO_2 as previously described[21]. Bacteria grown in 10 mL of Brucella-serum medium in 100-mL conical flasks following sub-culture were subjected to co-culture with AGS cells. Bacterial growth was measured by determining absorbance at 600 nm (A_{600}) with a spectrophotometer (GENEQUANT pro, Amersham Pharmacia Biotech), and colony forming units were determined for bacterial viability. Additional clinical isolates, 77 American and 51 Japanese isolates endoscopically obtained from 48 and 51 patients, respectively, were cultured on Brucella-serum agar at 37 ℃ in an atmosphere containing 10% CO_2. Isolates from different regions in the stomach, such as the antrum and corpus, were also included in 29 of 77 American isolates. The bacterial genomic DNA extracted by a DNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions were subjected to polymerase chain reaction (PCR) with specific primer pairs to examine *cdrA* gene diversity.

AGS cell culture conditions

AGS cells (human gastric adenocarcinoma epithelial cell line, CRL1739c) were grown in RPMI 1640 + 7 medium including streptomycin (20 μg/mL) and kanamycin (60 μg/mL) (Nikken bio medical Laboratory, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest, France) at 37 ℃ in an atmosphere containing 5% CO_2. AGS cells were seeded at a density of 0.5 × 10^6 cells in six-well plates (SUMITOMO BAKELIFE, Japan) and grown to about 80% confluence prior to co-culture experiments.

Co-culture of AGS cells with H. pylori strains

Subconfluent AGS cells (1 × 10^6) cultured in RPMI 1640 + 7 medium supplemented with 10% serum were washed twice by phosphate-buffered saline (PBS) and subsequently cultured with or without each *H. pylori* strain (HPK5, HPKT510 and HPK5CA) at a multiplicity of infection (MOI) of 150 or 300 in 1.5 mL of RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) alone for 48 h at 37 ℃ in an atmosphere containing 10% CO_2. The supernatant was collected at various times (3-48 h), centrifuged at 7000 r/min for 5 min to pellet bacteria and AGS cells, and subjected to sandwich enzyme-linked immunosorbent assay (ELISA) to measure IL-8 productions. AGS cells after co-culture were collected at the appropriate time and used in the following analyses.

Preparation of whole-cell and nuclear extracts

AGS cells were washed five times in ice-cold PBS, incubated with lysis buffer containing 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF, 100 μmol/L sodium orthovanadate, 10 μmol/L phenylmethylsulfonyl fluoride (PMSF) and a commercially available protease inhibitor mixture tablet (Complete, Roche Molecular biochemical, Indianapolis, IN), harvested by scraping and transferred to a microcentrifuge tube. Debris was removed by centrifugation at 10 000 *g* for 10 min (TOMY MRX-150) to collect the total proteins for whole-cell extracts. Nuclear proteins were prepared by Cellytic Nu-CLEAR Extraction Kit (SIGMA, Japan) according to a previous report[22]. Briefly, AGS cells were incubated with lysis buffer (10 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DTT and 0.25 mmol/L PMSF) on ice for 15 min. The pellet precipitated containing nuclei following centrifugation for 30 s at 10 000 *g* was resuspended in 100 μL extraction buffer (20 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol, 1 mmol/L DTT and 0.25 mmol/L PMSF) for 30 min at 4 ℃ with agitation. After centrifuging for 5 min at 20 000 *g*, the supernatant (nuclear fraction) was collected for nuclear extracts.

Immunoprecipitation

One milligram of the lysate proteins was incubated with polyclonal rabbit anti-*H. pylori* CagA antibody (Austral Biologicals, CA, United States) for 1 h at 4 ℃, followed by an overnight incubation with a 20 μL aliquot of Protein G Plus-agarose beads (Santa Cruz, United States) at 4 ℃, as previously described[20]. Briefly, the beads were washed with lysis buffer and boiled for 10 min in 2 × electrophoresis sample buffer (50 mmol/L Tris (pH 6.8), 10% sodium dodecyl sulfate, 12% 2-mercaptoethanol, 20% (wt/vol) glycine and 1% (v/v) bromophenol blue) to elute the immunoprecipitated proteins.

Western blotting analysis

For detection of CagA and its phosphorylation, equivalent amounts of the immunoprecipitated proteins (100 μg) were resolved by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred onto nitrocellulose membranes (Millipore Corporation, United States) by a semidry blotting apparatus KS-8460 (System Instruments Co., Ltd. Tokyo). The blots were blocked overnight with 4% (wt/vol) dried skim milk in Tris-buffered saline with Tween 20 at room temperature and then incubated with monoclonal antibody PY99 (diluted 1/1000; Santa Cruz, United States) for 1 h and anti-mouse IgG peroxidase-linked species-specific whole secondary antibody (diluted 1/1000; GE Healthcare Biosciences, Co. Ltd., United Kingdom) for 1 h to detect phosphorylated tyrosine protein. Immunodetection was performed by enhanced chemiluminescence Plus Western Blotting Detection Reagents (GE Healthcare Biosciences). Next, blots were stripped, reprobed with a specific polyclonal rabbit anti-*H. pylori* CagA antibody (diluted 1/1000; Austral Biologicals. CA, United States) and incubated with a horseradish perox-

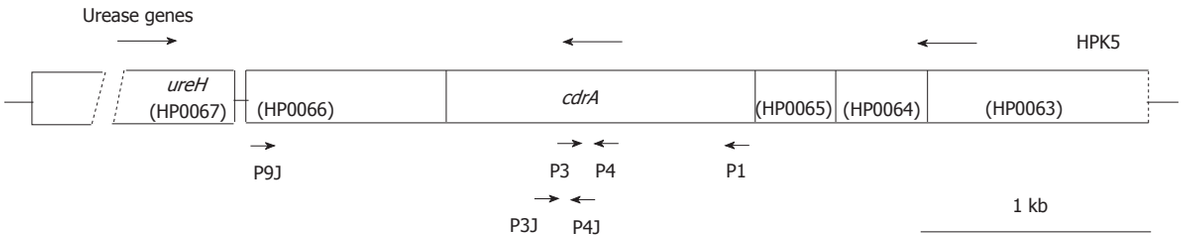

Figure 1 Location of primers used for cell division-related gene A in the region downstream of the urease gene cluster of *Helicobacter pylori* HPK5. Arrows above and below the map depict the direction of transcription and primers, respectively. The open reading frames (HP0063 to HP0067) are shown based on strain 26695.

idase-conjugated goat anti-rabbit secondary antibody (diluted 1/10 000; Jackson ImmunoResearch, PA, United States)[20]. Each 10 μg of nuclear and total proteins was subjected to 12% SDS-PAGE for detection of NF-κB and inhibition kappa B (IκB), respectively. Procedures of western blot analysis were followed as described above. Rabbit anti-human-NF-κB (p65) (diluted 1/200; Cell Signaling Technology, United States) and rabbit anti-human-IκB antibodies (diluted 1/200; Santa Cruz Biotechnology Inc, United States), and an anti-rabbit IgG antibody-HRP (diluted 1/2000; Santa Cruz Biotechnology Inc, United States) were used as primary and secondary antibodies, respectively. For a standard control, a rabbit anti-human-ERK2 antibody (diluted 1/200; Santa Cruz Biotechnology Inc, United States) was used to detect unphosphorylated ERK2 to confirm equal protein load[23]. The analyses were performed for least three independent experiments.

Measurement of IL-8 secretion from AGS cells

The amount of IL-8 secreted into culture medium after co-culture with *H. pylori* strains was determined by ELISA using the CytoSets system (BioSource International) according to the manufacturer's instructions. Fifty μL aliquots of the supernatant was briefly centrifuged at 7000 r/min for 10 min to remove bacteria and AGS cells, after which supernatants were added to an equal volume of regent on the 96-well ELISA plate. The absorbance of samples was measured at 550 nm using a 96-well microplate reader (Multiskan JX ver1.1, Thermo Labsystems, Finland), and the data was expressed as pg/mL. All samples were measured in triplicate in at least three independent experiments.

Measurement of mucosal IL-8 secretion

Frozen gastric antral biopsy specimens from 25 clinical patients infected with *H. pylori* were homogenized in 1 mL of PBS, and supernatants obtained by centrifugation were used for determination of IL-8 proteins by ELISA, as described previously[24]. Cytokine concentrations in homogenates were normalized in terms of total protein concentration of the biopsy specimen and were expressed as pg/mL.

PCR for cdrA gene diversity

To compare the *in vivo* IL-8 level in the biopsy specimens between the patients infected with *cdrA*-positive or -negative strains, PCR was utilized to examine the *cdrA* status in clinical isolates. The genomic DNA of clinical isolates, including 77 American and 51 Japanese strains, were subjected to PCR with specific primers for the *cdrA* gene of the HPK5 strain such as P1 (forward), P3 (reverse) and P4 (forward), previously described[17]. Additional new primers, P9J (reverse), P3J (reverse) and P4J (forward), based on the sequence of the *cdrA* gene of J99 strains, were also utilized in this study. The sequence of primer P1 was identical in strains, HPK5 and J99. The conditions used for PCR to amplify the 194-bp or 336-bp products in the central region of *cdrA*, which is highly conserved among strains using P3-P4 or P3J-P4J primer pairs, respectively, were as follows: pre-heat for 2 min at 96 ℃, followed by 40 cycles of 96 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30 s. To amplify the N-terminal region of *cdrA* using P1-P3 or P1-P3J primer pairs, PCR was performed with the following conditions: pre-heat for 2 min at 96 ℃, followed by 40 cycles of 96 ℃ for 30 s, 52 ℃ for 30 s, and 72 ℃ for 2.5 min. PCR was carried out at least two times with all sets of primer pairs, P1-P3, P1-P3J, P3-P4 and P3J-P4J, for determining the standing of the *cdrA* gene. In particular, for the strains representing no PCR product using various primer pairs mentioned above, P9J-P4 or P9J-P4J primer pairs were used to confirm the existence of the region from central to C-terminal on *cdrA* corresponding to flanking the downstream region of urease gene cluster. The PCR conditions were as follows: pre-heat for 2 min at 96 ℃, followed by 40 cycles of 96 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min. The location of these primers used for *cdrA* amplification (Figure 1) and primer sequences are shown (Table 1).

Statistical analysis

Fischer's exact test and the χ^2 test with Yates' continuity correction were applied using SPSS version 10.0 for Windows to compare differences on the level of IL-8 productions induced by *cdrA*-positive and -negative *H. pylori* strains. P value of < 0.05 were considered significant.

RESULTS

IL-8 secretion from AGS cells

To determine the effect of host response to *H. pylori*

Table 1 The sequence of primers used and the target region of cell division-related gene A amplified with combination of primers

Primers	Sequence (5′-3′)	Reference	Combination of primers	Target region of cell division-related gene A
P1 (forward)	TGAAGCACACGAAAGGA	17	P1-P3	N-terminal to central
P3 (reverse)	CATGCTCTAAAATCGTCG	17	P1-P3J	N-terminal to central
P3J (reverse)	ATCACCACGATCAGTCTG	This study	P3-P4	Central
P4 (forward)	TTTTGAGATCGGTGAAGC	17	P3J-P4J	Central
P4J (forward)	AACCACACGCTCATTTGC	This study	P4-P9J	Central to C-terminal
P9J (reverse)	GCTGAAAGGCCTGAATTCAG	This study	P4J-P9J	Central to C-terminal

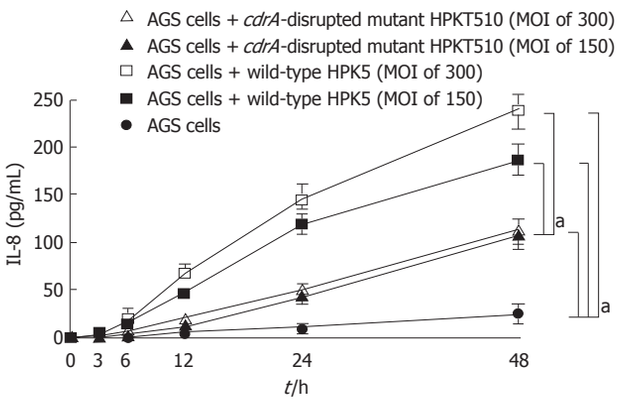

Figure 2 Interleukin-8 production from AGS cells induced by either wild-type or cell division-related gene A-disrupted mutant strains. [a]$P < 0.01$.

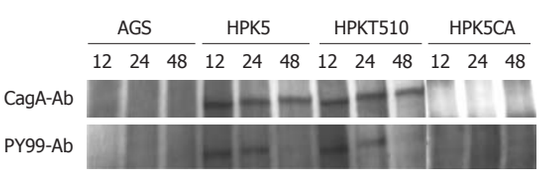

Figure 3 Cytotoxin-associated gene A status in AGS cells co-cultured with or without *Helicobacter pylori* strains. The immunoprecipitated proteins with anti-*Helicobacter pylori* (*H. pylori*) cytotoxin-associated gene A (CagA) antibody (CagA-Ab) were subjected to Western blotting with CagA-Ab (upper) and PY-99 antibody (PY99-Ab) (bottom), respectively. The assay was carried out with strains at MOI of 150. HPK5: Wild-type; HPKT510: Cell division-related gene A-disrupted mutant; HPK5CA: *cagA*-disrupted mutant.

during persistent infection in the human stomach, *in vitro* levels of IL-8 production from AGS cells co-cultured with HPK5 or HPKT510 strains were examined relative to time (Figure 2). Both strains induced the secretion of IL-8 from AGS cells and the levels increased time-dependently, but HPKT510, the *cdrA*-disrupted mutant, showed significantly lower levels, corresponding to 50%-60% of levels induced by wild-type HPK5 at MOI of 150 ($P < 0.01$). Furthermore, the levels of IL-8 were increased dose (MOI)-dependently by HPK5, but not HPKT510, which showed identical levels between MOI of 150 and 300.

Western blotting for CagA

Ultrastructural analyses revealed that the HPKT510 strain had a slightly wider periplasmic space between the inner and the outer membrane than that of HPK5[16]. To determine whether such morphological difference affects translocation of CagA into AGS *via* type Ⅳ secretory component system (T4SS) and its phosphorylation, which are considered to be risk factor for development of gastroduodenal disease, western blotting following immunoprecipitation with anti-*H. pylori* CagA antibody was carried out. CagA was detected in AGS cells co-cultured with HPK5 or HPKT510 strains, but no CagA was observed in AGS alone or when co-cultured with a *cagA*-disrupted mutant, HPK5CA as a negative control (Figure 3). The intensities of each CagA band are similar between both strains. Phosphorylation of CagA was detected up to 24 h after co-culture with HPK5 or HPKT510 strains and had similar intensity, indicating that there were no differences in the status of CagA between both strains.

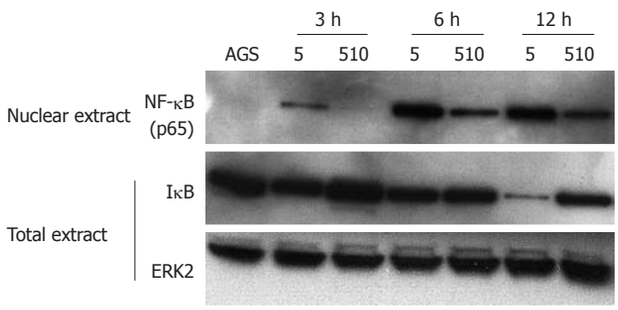

Figure 4 Detection of nuclear factor kappa B (p65) (upper), inhibition kappa B (middle) and extracellular signal-regulated kinase 2 (bottom) in nuclear and total extracts, respectively at 3, 6 and 12 h after being co-cultured with *Helicobacter pylori*. Unphosphorylated extracellular signal–regulated kinase 2 (ERK2) was detected as the control in this study[23]. Molecular weights of nuclear factor kappa B (NF-κB), inhibition kappa B (IκB) and ERK2 are 65, 35-37 and 42 kDa, respectively. The assay was carried out with strains at a MOI of 150. AGS: AGS cells co-cultured without *H. pylori*; HPK5: AGS cells co-cultured with wild-type HPK5; HPKT510: AGS cells co-cultured with *cdrA*-disrupted mutant HPKT510.

Western blotting for NF-κB (p65) and IκB

To determine how the expression level of NF-κB transcription factor affects the expression of many genes involved in inflammation and immunities, western blotting was carried out utilizing AGS cells co-cultured with HPK5 or HPKT510 strains for 3, 6 and 12 h. Overall, NF-κB levels in the nuclear extracts from AGS cells with HPKT510 were definitely lower compared to those with HPK5, whereas IκB levels in total extracts were found to be higher in HPKT510 (Figure 4), indicating that activation of NF-κB was relatively repressed in AGS cells stimulated by *H. pylori* with disruption of *cdrA*.

Table 2 ***Helicobacter pylori*** **cell division-related gene A status (positive or negative) and genotype (allele type) in clinical isolates *n* (%)**

H. pylori cdrA			
Status	**Genotype**	**United States (48 patients)[1]**	**Japan (51 patients)**
Positive	Type I	0 (0)	4 (7.8)
	Type Ⅱ	13 (16.9)	46 (90.2)
Negative	Type Ⅲ	29 (37.7)	1 (2.0)
	Type Ⅳ	35 (45.4)	0 (0)
Total		77[2]	51

H. pylori cdrA: *Helicobacter pylori* cell division-related gene A. [1]In 29 out of 48 patients, each isolate was obtained from both antrum and corpus in same stomach; [2]The total 77 isolates include 44 and 33 isolates obtained from antrum and corpus, respectively.

Table 3 Comparison of cell division-related gene A status and genotype (allete type) between antrum and corpus isolates in 29 American patients

	***cdrA* status[1]**		***cdrA* genetype (allele type)**		
cdrA positive (6 cases)	Same[2]: 1	Antrum (+)[3] Corpus (+)	Identical[4]: 1	Antrum Type Ⅱ	Corpus Type Ⅱ
	Difference: 5	Antrum (+) Corpus (-)	Difference: 5	**4 Type** Ⅱ	**Type** Ⅲ
				1 Type Ⅱ	**Type** Ⅳ
cdrA negative (23 cases)	Same: 22	Antrum (-) Corpus (-)	Identical: 16	5 Type Ⅲ	Type Ⅲ
				11 Type Ⅳ	Type Ⅳ
	Difference: 1	Antrum (-) Corpus (+)	Difference: 7	4 Type Ⅲ	Type Ⅳ
				2 Type Ⅳ	Type Ⅲ
				1 Type Ⅳ	**Type** Ⅱ
Total (%)	Same: 23 (79)		Same: 17 (59)		
	Difference: 6 (21)		Difference: 12 (41)		

[1]The status of cell division-related gene A (*cdrA*) determined with isolate from antrum was divided to two category groups (*cdrA* positive and negative); [2]Same or difference: *cdrA* status is same or difference between isolate (antrum and corpus); [3]+: *cdrA* positive isolate; -: *cdrA* negative isolate; [4]Identical or difference: the genotypes of *cdrA* is identical or difference between isolate (antrum and corpus). Bold denotes the 6 cases representing differences at *cdrA* status and genotype between isolates (antrum and corpus).

Genetic diversity of H. pylori cdrA

To examine the *cdrA* status [category such as *cdrA*-positive and -negative, including 4 allele types (Ⅰ to Ⅳ)] in 128 clinical isolates, PCR was performed with appropriate specific primer pairs. In a previous report, PCR with P1-P3 yielded the 731 bp (short fragment) and approximately 2 kb (long fragment) in HPK5 and other three strains examined respectively, demonstrating that the shorter product of HPK5 was due to partial deletion in the N-terminal region of *cdrA*. However, *cdrA* was functionally involved in cell division, morphology, long-term survival and susceptibilities to beta-lactam and its transcript could be detected[16,17]. On the other hand, the 194-bp amplified in the central region of *cdrA* was detected in all strains, indicating that size diversity exists within the N-terminal region of *cdrA* among individual strains[17]. In this study, 128 clinical isolates (77 American and 51 Japanese) were subjected to PCR with N-terminal primer pairs (P1-P3 and P1-P3J) which demonstrated that the short fragment (allele type Ⅰ) was obtained in only 4 Japanese isolates (7.8%) including HPK5 and the long fragment (allele type Ⅱ) was detected in 13 American (16.9%) and 46 Japanese (90.2%) isolates. In addition, no product was observed in the 64 American (83.1%) and 1 Japanese (2.0%) isolates by PCR with central region primer pairs (P3-P4 and P3J-P4J). Of the 64 American isolates showing no amplification on the central region, 35 isolates have no product amplified on the C-terminal region with P9J-P4J and P9J-P4 pairs. The results revealed that the downstream region of the urease gene cluster, including *cdrA,* was absent in the 35 isolates (allele type Ⅳ). However, PCR product on the C-terminal region with P9J-P4J or P9J-P4 pairs was detected in remaining 29 isolates, demonstrating that they have partial *cdrA* sequence loss between the N-terminal and central regions (allele type Ⅲ). Taken together, the status of the *cdrA* gene among individual isolates examined was divided into two categories; *cdrA*-positive including allele types Ⅰ (HPK5) and Ⅱ (J99 and 26695), and *cdrA*-negative including allele types Ⅲ and Ⅳ (Table 2).

Comparison of cdrA status between antrum and corpus

The *cdrA* allele type of each isolate from the antrum and corpus in the stomach of 29 American patients demonstrated that *cdrA* allele between the regions was identical in 17 patients (59%); however, 9 of the remaining 12 patients possessing a different allele had a isolate with more loss of the *cdrA* sequence in the corpus compared to the antrum (Table 3). Regarding the status of *cdrA*, 6 out of 29 patients had different *cdrA* status between the regions, and of which, 5 patients harbored *cdrA*-positive and -negative isolates in the antrum and corpus, respectively (Table 3).

Measurement of mucosal IL-8 secretion

The *cdrA*-disrupted mutant HPKT510 showed significantly lower levels of IL-8 secretion *in vitro* compared to wild-type HPK5. To investigate whether *cdrA* is associated with IL-8 production *in vivo*, mucosal IL-8 secretion of biopsy specimens was measured using samples obtained from 20 American patients infected with *cdrA*-negative and 5 American patients with *cdrA*-positive *H. pylori*. Measurement of IL-8 in these specimens demonstrated that the *cdrA*-negative group exhibited lower IL-8 levels than those of *cdrA*-positive group. The average value of IL-8 in the *cdrA*-negative group was 111 pg/mL, corresponding to the average of 60% in the *cdrA*-positive group (156 pg/mL). This tendency was consisted with *in vitro* data, revealing that *cdrA*-negative *H. pylori* does not induce IL-8 secretion as strongly in the human stomach as *cdrA*-positive strains.

DISCUSSION

H. pylori colonizes more than half of the world's population. While it is clear that this organism induces a strong innate and adaptive immune response leading to active inflammation in the gastric mucosa, the ability of *H. pylori* to establish persistent infection so efficiently has not been fully elucidated. *H. pylori* possesses a number

of virulence factors, and some, such as urease, flagella and lipopolysaccharide (LPS), contribute to its persistence[25-27], whereas others, such as CagA and vacuolating cytotoxin, appear to confer increased virulence[28,29]. Furthermore, the strain-specific genes found outside of the *cag* pathogenicity island, especially genes in the plasticity regions[30] and genes with variable structures/genotypes, have considerable interest in their contribution to pathogenesis and the host immune response. Nearly half of the strain-specific genes of *H. pylori* are located in the plasticity regions in strains 26695[31] and J99[30,32]. There is evidence that genetic recombination among *H. pylori* may occur during the course of infection in the stomach. The diversity or phase variation in *H. pylori* genes such as *babA*[33] and *fucT1*[26] likely contributes to the evasion of the host immune response and thereby has a role in the establishment of persistent infection in the stomach. Based on the genomic information of the sequenced *H. pylori* strains 26695[31] and J99[32], more than a third of *H. pylori* genes have unknown function, suggesting that there may be strain-specific genes such as *ctkA*[34] involved in the mechanisms of persistence as well as the host immune response.

One of unique genes found in the HPK5 strain, *cdrA* located in the downstream region of urease gene cluster, has not only a inhibitory role for cell division, but is also involved in elongation and death of cells *via* cell wall synthesis at the site of division[16,17]. Furthermore, the *cdrA*-disrupted mutant HPKT510 had a longer survival time compared to the wild-type HPK5 in both liquid and solid media, as well as in serum-free medium and aerobic conditions[16]. Loss of the *cdrA* gene during infection is frequently found in a mouse infected with *H. pylori* strain B128[18] and in the human stomach infected with J99[19]. The present study found that *cdrA*-negative strains resulted in lower levels of IL-8 production *in vitro* and *in vivo* compared to *cdrA*-positive strains. *In vitro* data showed that increased IL-8 production was dose-dependently observed by *cdrA*-positive HPK5, but not *cdrA*-disrupted HPKT510. In addition, nuclear accumulation of NF-κB in AGS co-cultured with HPKT510 was lower compared to HPK5, suggesting that activation of the NF-κB signaling pathway was relatively repressed by stimulation of the *cdrA*-negative strain, which coincided with decreased IL-8 production. These indicated that *cdrA*-defective *H. pylori* may evade immune clearance due to limiting bactericidal effects of pro-inflammatory molecules leading to promotion for persistent infection, likely *via* repression of the NF-κB signaling pathway. Persistent infection was not observed in IL-10-deficient mice with strong inflammation, implying that attenuate host immunity is necessary for *H. pylori* colonization[35]. Accordingly, it is acceptable that *H. pylori* infection occurred in the stomach with immature immunity during infantile generation. The proper NF-κB transcriptional response is primarily regulated by post-translational modification of NF-κB signaling components. On the other hand, alternative splicing of NF-κB signaling components is another way to control the NF-κB signaling pathway[36,37]. Alternative splicing of some NF-κB signaling components can be induced by prolonged exposure to a NF-κB-activating signal, such as LPS, suggesting a mechanism for negative feedback to dampen excessive NF-κB signaling. In particular, alternative splicing events in Toll/interleukin-1 NF-κB signaling pathways *via* Toll like receptors can inhibit the NF-κB response[38]. This raises the possibility that changes in bacterial constituents concerned with disruption of *cdrA*, such as cell envelope components including LPS, may exert on the complicated NF-κB signaling pathway.

As HPKT510 had a slightly wider periplasmic space[16], the effector protein, CagA, was investigated to determine whether this morphological change altered the function of T4SS. The CagA production, its translocation and phosphorylation were indistinguishable between HPK5 and HPKT510 strains, indicating that such morphological differences related with inactivation of *cdrA* had no effect on CagA-associated pathogenicity, including CagA-related signaling pathway and T4SS. Cytoplasmic nucleotide-binding oligomerization domain (Nod) molecules, Nod1 and Nod2, have been shown to be specific ligands of diaminopimelic acid-containing muropeptides and muramyl dipeptide, respectively[39,40], and were important components of the innate immune response. A peptidoglycan-derived muropeptide possessing a Nod1 motif was translocated through T4SS and affected cell signaling *via* activation of NF-κB in the host cell, leading to the stimulation of an intracellular pro-inflammatory response[41]. In terms of conserved domains, part of HPK5 *cdrA* belongs to the SM1-NRK4 family, beta glucan and cell wall synthesis enzyme family, and another belongs to the SpoIIE-FtsK-ATPase family. The properties of penicillin-binding proteins such as PBP1, PBP2, PBP3 and PBP4 varied between HPK5 and HPKT510 strains[16], suggesting that an alteration of such peptidoglycans influences the level of IL-8 production *via* the Nod1-NF-κB signaling pathway. In fact, the measurement of *nod1* transcript level in AGS by real-time RT-PCR documented that HPKT510 induced lower level of *nod1* transcript compared to HPK5 (data not shown), which supports the Nod1-NF-κB pathway involved in the difference of IL-8 production.

The *cdrA* status in almost all Japanese isolates were *cdrA*-positive (98%), but not in isolates obtained from Americans, demonstrating that American isolates had a greater diversity and higher prevalence of *cdrA*-negative strains (83.1%). Evaluation of *cdrA* status in isolates obtained from different regions of the stomach revealed that in 5 of the 6 cases possessing different status between the antrum and corpus of the stomach, the *cdrA*-negative isolate was from the corpus, suggesting that a *cdrA*-negative strain might be more adaptable to colonize in the corpus over a longer time period than the *cdrA*-positive strain. A *cdrA*-disrupted mutant could survive

longer under the stresses, such as presence of beta-lactam antibiotics, serum-free and aerobic conditions, than the wild-type strain[16]. It is possible that changes in biological behavior accompanied by inactivation of *cdrA* are necessary to stay balanced for the establishment of long-term infection in the human stomach. When the HPK5 *cdrA* sequence was compared to reference strain 26695[31], the *cdrA* sequence was a fusion of the ~130 codon hp0064 gene to the middle ~one third of the hp0066 gene, with deletion of the interstitial hp0065 and first part of hp0066. A similar fusion is evident with respect to the reference strain J99 (jhp0059 and jhp0061) or HPAG1 (hpag1_0064 and hpag1_0067)[42]. These two reference strains might represent the majority class in the Japanese population, which gave 2 kb instead of 730 bp PCR products. In contrast, two other sequenced strains, Shi470[43] and G27[44], seem to contain only the ~130 codon ORF, not the longer one, and thus might possibly represent one of the major classes in the United States population. The HPK5 type *cdrA* stems from gene fusion and synthesizes a partially defective protein that impacts cell wall synthesis or structure and a greater release of peptidoglycan fragments, which can be proinflammatory through the Nod1-NF-κB pathway. More studies are required in the future to dissect the mechanistic role of *cdrA* and the alteration of comprehensive genomix/proteomix affected by inactivation of *cdrA*. Analysis for putative deletion alleles might actually provide us with insights to elucidate whether the phenomenon resulted from such sources as large insertions or genome rearrangements.

In histological observation, as far the confined tissue sections of small biopsy specimens were examined, no significant finding was observed in the gastric mucosa colonized with either *cdrA*-positive or *cdrA*-negative *H. pylori* strains. We may need more examinations in detail with extended sections and specimens to confirm the differences in histological findings, including inflammation with neutrophil infiltration.

We concluded that the presence of *H. pylori cdrA* was associated with effective production of pro-inflammatory cytokine, IL-8, both *in vitro* and *in vivo*. Therefore, *cdrA*-inactivated *H. pylori* strains may result in attenuated host immunity and evade immune clearance due to repression of the NF-κB signaling pathway in the host cell, leading to persistent infection. Finally, our studies suggest that *cdrA*-negative *H. pylori* strains are more likely to colonize in the corpus over long time periods compared with *cdrA*-positive strains.

COMMENTS

Background

Helicobacter pylori (H. pylori) infection up-regulates secretion of various inflammatory cytokines including interleukin-8 (IL-8), whose production *in vitro* and *in vivo* is recognized as a host response to microbes. The levels of IL-8 production differ among individual strains, suggesting the existence of unique genes involved in host response and genetic diversity during infection in the stomach. Furthermore, the effect of fundamental constituents belonging to cell division of *H. pylori* on host response is unclear.

Research frontiers

H. pylori infection and its persistence in the stomach is the most important event to actually lead a variety of diseases. However, the functions and practices of the cell division of strains colonized in an individual stomach remain unclear. Furthermore, how the bacterial behaviors concerned with the host response is still unknown. In this study, the authors demonstrate that *H. pylori* cell division-related gene A (*cdrA*) required for cell division and morphological shape could be a potential role for mediating IL-8 secretion.

Innovations and breakthroughs

Reports have highlighted the importance of *H. pylori*-host interaction to understand host immunity and pathogenesis. Certain molecules, such as cytotoxin-associated gene A (*CagA*) and outer membrane proteins, were shown to be involved in these phenomena; however, the relationship between cell division- and morphological shape-related molecules and host response associated with persistent infection in the stomach is unknown. This is the first study to report that *cdrA* influenced IL-8 secretion (*vivo* and *vitro*) and loss of *cdrA* may attenuate the host immunity due to repression of NF-κB, leading to persistence.

Applications

In this study, how *H. pylori cdrA* functions and influences bacterium-host interaction was investigated, which provides insights into understanding bacterial fundamental components and their effect on the stomach, including host response, and a future strategy for therapeutic intervention in the treatment of patients with *H. pylori* infection.

Terminology

H. pylori cdrA, one of the unique genes discovered in strain HPK5, has not only a repressive role on cell division but is also involved in cell elongation and cell death *via* cell wall synthesis (mainly penicillin binding proteins) at the division site. The *cdrA*-disrupted mutant is able to survive for long time periods and is more resistant to the bactericidal of beta-lactam antibiotics than the wild-type. The *cdrA* gene tends to be lost during infection to facilitate adaption in the stomach, resulting in a persistent infection.

Peer review

This interesting manuscript showed that the presence of *H. pylori cdrA* was associated with the effective production of IL-8 compared to the inactivation of *cdrA in vitro* and *in vivo*. Additionally, they observed that *cdrA*-inactivated *H. pylori* strains may result in attenuated host immunity and evade immune clearance due to repression of the NF-κB pathway, leading to persistent infection.

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ORIGINAL ARTICLE

A *Macaca mulatta* model of fulminant hepatic failure

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Abstract

AIM: To establish an appropriate primate model of fulminant hepatic failure (FHF).

METHODS: We have, for the first time, established a large animal model of FHF in *Macaca mulatta* by intraperitoneal infusion of amatoxin and endotoxin. Clinical features, biochemical indexes, histopathology and iconography were examined to dynamically investigate the progress and outcome of the animal model.

RESULTS: Our results showed that the enzymes and serum bilirubin were markedly increased and the enzyme-bilirubin segregation emerged 36 h after toxin administration. Coagulation activity was significantly decreased. Gradually deteriorated parenchymal abnormality was detected by magnetic resonance imaging (MRI) and ultrasonography at 48 h. The liver biopsy showed marked hepatocyte steatosis and massive parenchymal necrosis at 36 h and 49 h, respectively. The autopsy showed typical yellow atrophy of the liver. Hepatic encephalopathy of the models was also confirmed by hepatic coma, MRI and pathological changes of cerebral edema. The lethal effects of the extrahepatic organ dysfunction were ruled out by their biochemical indices, imaging and histopathology.

CONCLUSION: We have established an appropriate large primate model of FHF, which is closely similar to clinic cases, and can be used for investigation of the mechanism of FHF and for evaluation of potential medical therapies.

Key words: Fulminant hepatic failure; *Macaca mulatta*; Biochemistry; Imaging; Pathology

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INTRODUCTION

Fulminant hepatic failure (FHF) is an uncommon and challenging clinical disease characterized by sudden and severe hepatic injury and dysfunction, with many different symptoms and complications, and high mortality. The term was first used in 1970 to describe a potentially reversible disorder that was the result of severe liver injury in the absence of pre-existing liver disease, with an onset of encephalopathy within 8 wk of symptom appearance[1]. The incidence and etiology of FHF vary according to different geographic regions[1,2], and viral hepatitis and drug or toxin ingestion are the most common causes. As the pathophysiological changes are complicated and still need to be investigated, a clinically relevant large-animal model would be an indispensable tool. Orthotopic liver transplantation has been proved to be an effective treatment[3-6], but the shortage of donor organs has restricted its wide application[7-9]. The development of new therapies, including hepatocyte transplantation, stem cell transplantation, tissue engineered liver and bioartificial liver support systems, is still under investigation[3-6]. Before these treatments can be used clinically, their effects should be determined in large animal models. For a satisfactory animal model of FHF, seven criteria were suggested by Terblanche *et al*[10] and Fourneau *et al*[11]: (1) reversibility; (2) reproducibility; (3) death from liver failure; (4) presence of a therapeutic window; (5) a large animal model; (6) minimal hazard to personnel; and (7) a conscious animal model. Previous reports have described different animal models of FHF induced by various methods[7,12,13], but all the established models did not entirely satisfy all these criteria. Moreover, selection of a species with similar metabolic and physiological properties to humans is of importance. In this article, we describe, for the first time, the establishment of a *Macaca mulatta* model of FHF and the dynamic biochemical, pathological and imaging changes are also described.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma-Aldrich. Inc., St. Louis, MO. α-amanitin was from Alexis Biochemicals Co., Lausen, Switzerland. Gd-BOPTA (multihance) and SonoVue, contrast media for magnetic resonance imaging (MRI) and ultrasonography, respectively, were from Bracco Sine Pharmaceutical Co., Shanghai, China.

Animals

Two healthy female *Macaca mulattas*, 3 and 4 years old and with body weights of 5.5 kg and 4.6 kg, respectively, were purchased from the Safe and Secure Experimental Animal Breeding Base in Chengdu, China. They were housed in a large animal care facility with a constant temperature of 20 ℃ ± 1 ℃ and a 6 am to 6 pm light cycle. Two weeks were allowed for the animals to acclimatize to the animal facility before the study. They were fed with standard dry monkey food, washed apples and water *ad libitum*. Animal procedures and care were conducted in accordance with the institutional guidelines and in compliance with national and international laws and policies.

Study design

After premedication with ketamine (15 mg/kg im), skin preparation was performed on the posterior legs for blood collection from the saphenous vein and on the abdomen for intraperitoneal infusion and hepatic needle biopsy. LPS (1 μg/kg) and α-amanitin (0.1 mg/kg) were mixed in 50 mL physiological saline and slowly infused into the peritoneal cavity. Before their administration, blood biochemical parameters of the liver, kidney, heart and pancreas were measured, and total body MRI and ultrasonography were performed to acquire the physiological data as baseline values (expressed as "0" in the figures). After administration, blood biochemical parameters were measured every 12 h. An imaging examination was performed every 24 h.

Core needle biopsy of the liver guided by ultrasonography scan was performed 12 h after administration and repeated every 24 h. Part of the needle biopsy specimens was subjected to frozen section and oil red O staining.

The state of consciousness and behavior of the animals was observed and the hepatic encephalopathy was defined according to the West-Haven criteria in humans[14].

The two *Macaca mulattas* underwent necropsies after death, which included a full macroscopy and a histological evaluation of the liver, kidneys, lungs, heart, brain, spleen, pancreas and lymph nodes. Tissues were fixed in 10% neutral buffered formalin, sectioned at 5 μm and stained with hematoxylin and eosin (H and E).

RESULTS

Clinical features and animal survival

The animals showed a gradual increase in listlessness and loss of appetite after toxin administration. Anorexia and vomiting occurred 24 h after administration, followed by grasping disability, mental indifference, drowsiness and coma. Hepatic coma appeared at 42 h and 56 h, and decease occurred at 49 h and 70 h, respectively.

Biochemical results

We measured the liver enzymes, bilirubin and coagulation indices to determine the extent of liver injury. The data below were the average values from the two *Macaca mulatta* models when both were alive, or from one monkey when one died. After administration, the values of alanine aminotransferase and aspartate aminotransferase began to increase significantly and reached a peak at 36 h with over 300-fold and 200-fold increases from the baseline, respectively (Figure 1A), then the values sharply decreased. The total bilirubin, direct bilirubin and indirect bilirubin all increased gradually, and the final measured values exceeded a 30-fold rise from the corresponding

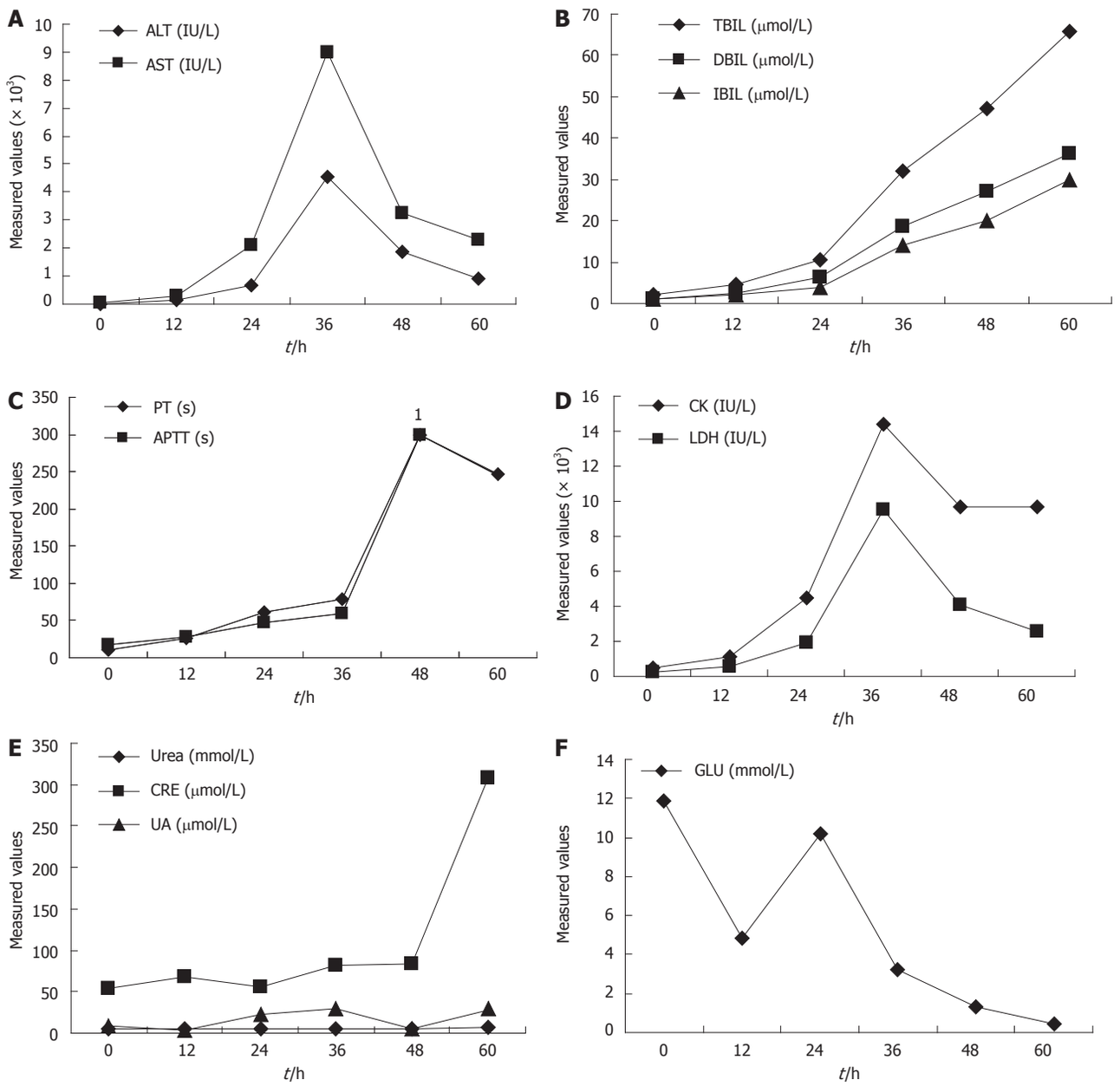

Figure 1 Blood biochemical parameters of the *Macaca mulatta* model of fulminant hepatic failure. The abscissa and ordinate represent measured time and value, respectively. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; PT: Prothrombin time; APTT: Activated partial thromboplastin time; CK: Creatine kinase; LDH: Lactate dehydrogenase; CRE: Creatinine; UA: Uric acid; GLU: Glucose; FHF: Fulminant hepatic failure. [1]Represents values > 300 s, which exceeded the range of the equipment, so we did not obtain an exact measurement.

baseline values (Figure 1B). The consistent increase in bilirubin and the notable decrease in enzymes after 36 h suggest the emergence of enzyme-bilirubin segregation. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were also prolonged after administration. At 48 h, both the levels exceeded 300 s so that we could not measure the exact number using the machine. Even the last two measured levels of PT and APTT showed little diminution, and at 250 s were still 14- to 22-fold of the baseline (Figure 1C). The ratios of prothrombin activity were 1.18% and 5.53%.

To determine whether the toxins damaged other organs, the biochemical parameters of the heart, kidneys and pancreas were measured. Creatine kinase (CK) and lactate dehydrogenase (LDH), markers of myocardial injury, also displayed peaks at 36 h with values 27-fold and 38-fold that of baseline, respectively (Figure 1D). Of the three kidney-associated parameters, i.e., urea, creatinine (CRE) and uric acid, only the level of CRE obviously increased after 48 h (Figure 1E). The blood glucose concentration decreased (Figure 1F), and the serum amylase of the pancreas did not show any significant changes.

Imaging

MRI and ultrasonography were performed every 24 h, and the final scans were performed when the animal

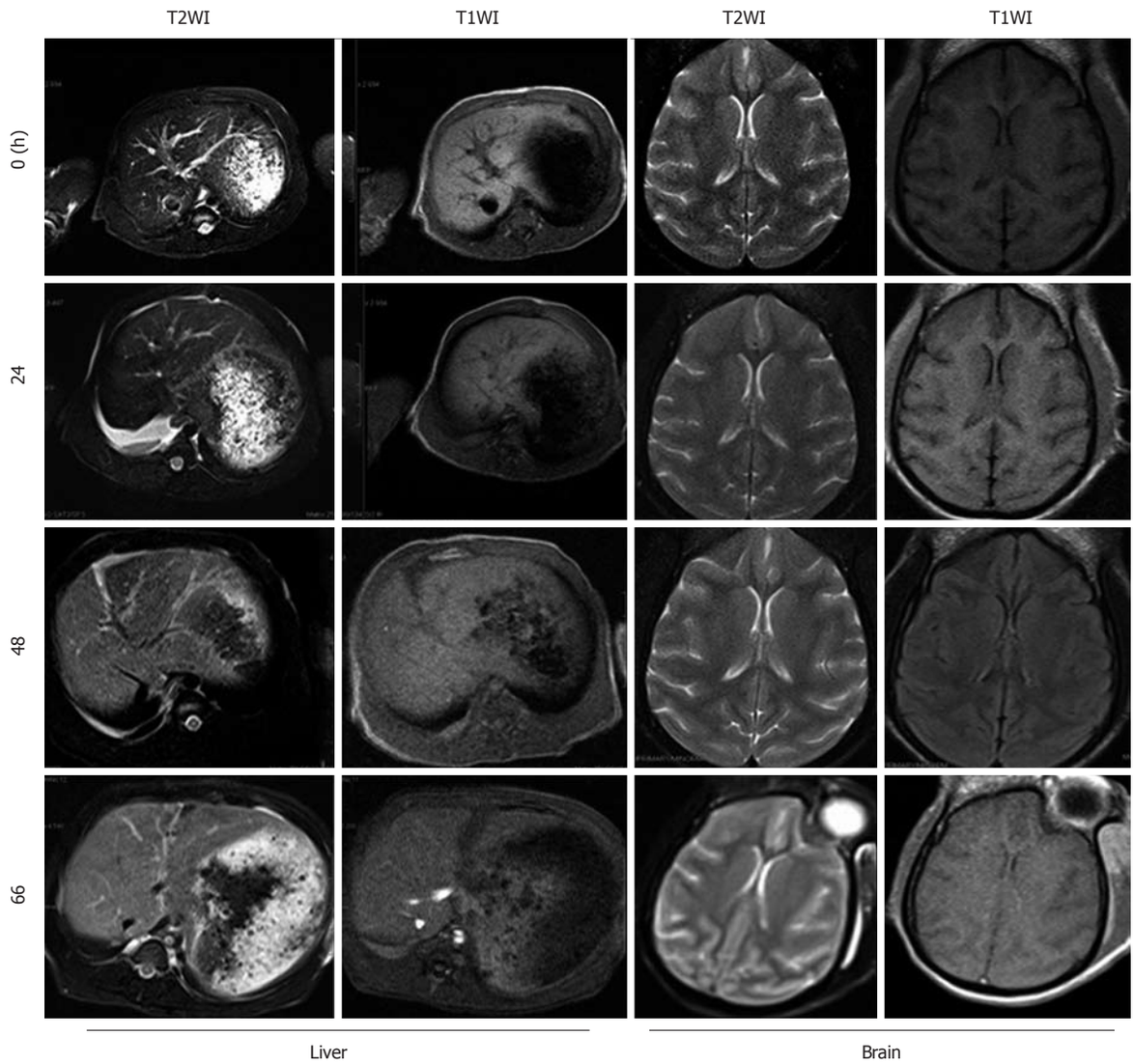

Figure 2 Magnetic resonance imaging of the liver and brain of *Macaca mulatta* model at different times. T2WI: T2-weighted imaging; T1WI: T1-weighted imaging.

died. At 24 h, MRI showed slight signals emerging in the left lobe of the liver, and Gd-enhanced T1-weighted imaging demonstrated homogeneous intensity. The signal began to increase heterogeneously in the parenchyma at 48 h, and became more heterogeneous and diffuse at 66 h, and the intensity decreased on a widespread basis in the hepatobiliary period (Figure 2). In ultrasonography, the echo of the liver parenchyma was slightly enhanced at 48 h, especially in the left lobe, and the contrast sonogram with SonoVue showed low perfusion. The hepatic artery resistance index increased. At 60 h, the echo was still enhanced slightly, but the portal vein velocity decreased and the hepatic artery resistance index increased further (Figure 3). Before 48 h, no predominant changes were found in brain MRI, but at 66 h, abnormal rhythmic signals emerged in the frontal lobe and both temporal lobes, which demonstrated hyperacute ischemia (Figure 2). We also scanned the thoracic and abdominal organs and no marked abnormality was found (data not shown).

Pathological changes

Core needle biopsies were performed at 12 h and 36 h after administration and the animals underwent necropsies after death. The main organs were evaluated, including the liver, kidneys, lungs, heart, brain, spleen, pancreas and lymph nodes, for macroscopic and histological changes.

H and E and oil red O staining showed that the liver developed severe steatosis at 36 h after toxin infusion, and in the necropsy liver, extensive parenchymal necrosis was found. The hepatic cord was dissociated, with disordered hepatocytes, and the hepatic sinusoid was extended with hyperemia. Vacuoles appeared in the cytoplasm, and patchy necrosis was found in the portal areas. In the non-necrotic areas, most hepatocytes were stained reddish-orange by oil red O. In the necropsy section, massive necrosis caused loss of almost all of the hepatocytes, and only the reticular structure remained, which is similar to the case in humans suffering from acute severe viral hepatitis (Figure 4).

With the naked eye, the livers appeared softer and smaller, with sharper and thinner edges, than the normal liver. The surface color was red and yellow, and it was a uniform yellow at the cut surface (Figure 4). Vascular dilatation and hyperemia were found on the surface of

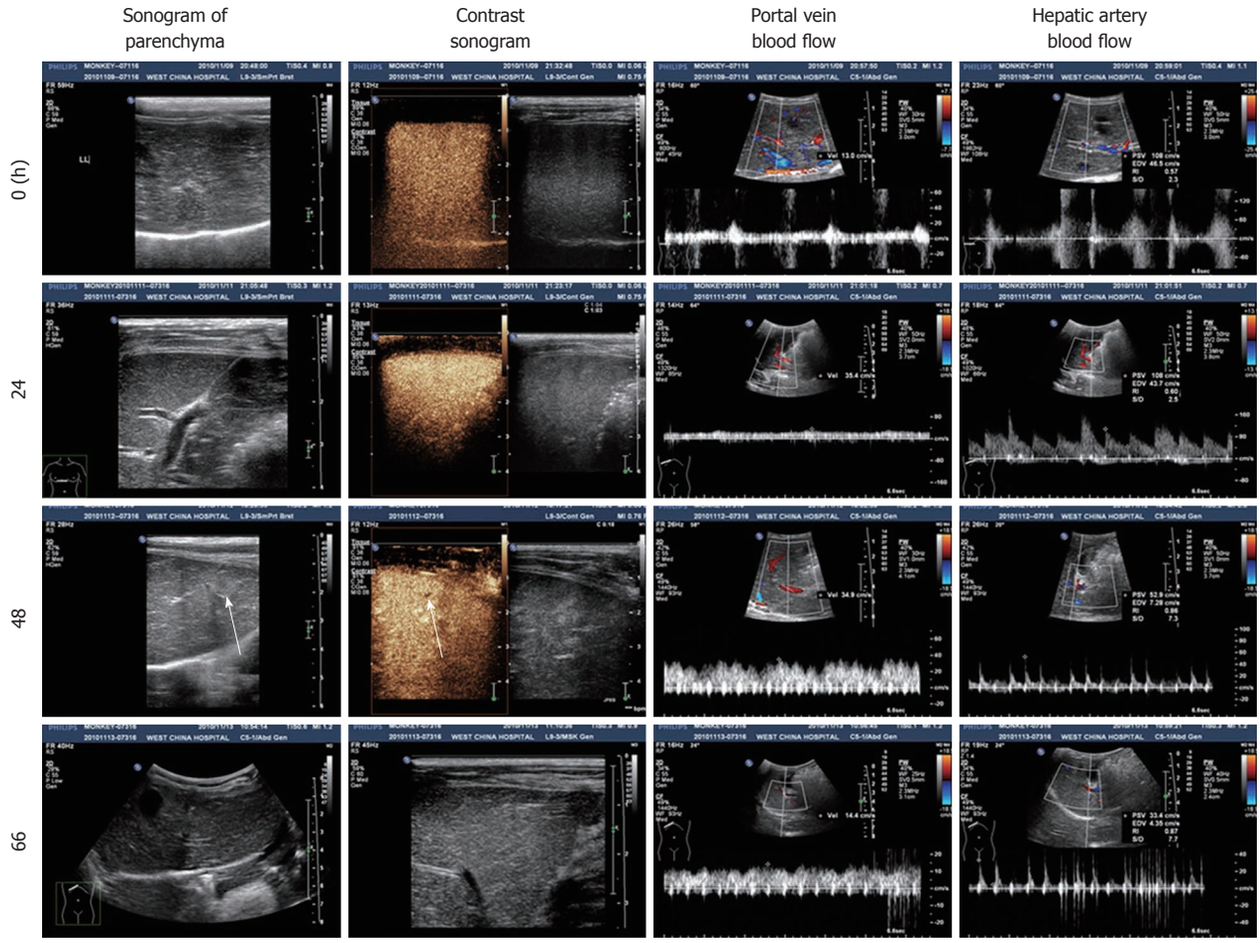

Figure 3 Liver sonogram of the *Macaca mulatta* model at different times. Arrows show a slightly enhanced echo and lower perfusion of SonoVue at 48 h.

the brain and cerebral edema was proved by widened gyri and shallowed sulci (Figure 5). Under microscopy, the superficial layer of the brain was loose, edematous and weakly stained (Figure 6).

Both kidneys showed hyperemia. The heart, spleen and pancreas did not display obvious gross changes (Figure 5). No peritoneal hyperemia, adhesions or edema in the peritoneal cavity was found. The lymph nodes located in the mesentery were enlarged and darkened. Under microscopy, they showed diffuse bleeding and lymphocytes were extensively reduced with much histiocyte proliferation. Arterioles in the lymphoid follicles were found to have hyaline degeneration. Both kidneys were also injured and demonstrated ectasia and hyperemia of the capillaries in the renal glomerulus and the stroma of the kidney tubules, and there was cellular swelling in the tubule epithelial cells. Only a wave-like change of the myocardium was found in the heart (Figure 6).

DISCUSSION

In this study, we have established, for the first time, a *Macaca mulatta* model of FHF, which satisfies all of the criteria for a large animal model of FHF proposed by Terblanche *et al*[10] and Fourneau *et al*[11], especially a conscious animal model for observation of the development of hepatic encephalopathy. All the clinical features and biochemical parameters, including liver enzymes, bilirubin as well as coagulation activity, confirmed that this model matched all the diagnostic criteria of human FHF. In addition, we dynamically examined and recorded the pathological and imaging changes in the liver and extrahepatic organs during disease progression.

Animal models of FHF are urgently needed to fully investigate the pathogenesis, progression, diagnosis and treatment of this serious disease clinically. Novel therapeutic strategies including hepatocyte transplantation, stem cell transplantation, tissue engineered liver and bioartificial liver support systems are under investigation. However, all the therapies need to be tested in an animal model before clinic use. Over the past 30 years, various animal models of FHF have been created with different methods[7,12,13], including models using rodents[15-18], dogs[19-23] and pigs[24,25]. However, differences in the metabolic and physiological properties in these distantly relative species restricted the application of the results to humans. As the closest relative to the human, the primate possesses much more similar metabolic and physiological properties, and a primate with FHF is considered to be the best large animal model for use in basic and pre-clin-

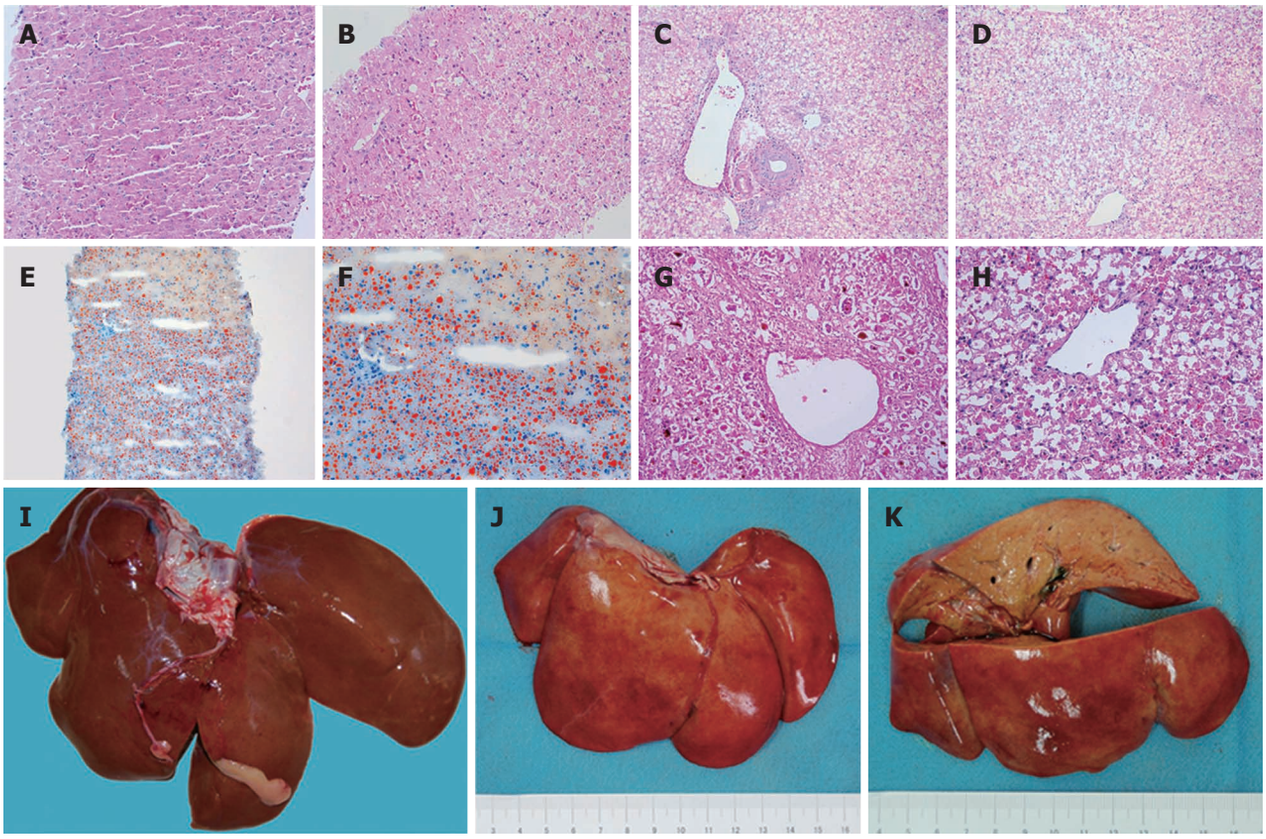

Figure 4 Histopathological changes of the liver. A: Tissue from needle biopsy at 12 h. Changes in organizational structure and cell morphology were not obvious [hematoxylin and eosin (H and E) stain, × 200]; B: Tissue from needle biopsy at 36 h. Vacuoles appeared in the hepatocellular cytoplasm (H and E stain, × 200); C, D: Tissue from necropsy after death at 70 h. Hepatocellular necrosis was distributed in the portal area (C) and central area (D) (H and E stain, × 200). Massive necrosis caused almost all of the hepatocytes to be lost, and only the support structure remained; E: Frozen tissue from needle biopsy at 36 h. The extensive reddish-orange color suggested serious fatty degeneration (oil red O stain, × 100); F: Frozen tissue from needle biopsy at 36 h. In the borderline between necrosis and steatosis, the reddish-orange color was obvious (oil red O stain, × 200); G, H: Comparison of pathological changes of fulminant hepatic failure in humans induced by viral hepatitis (G) and a *Macaca mulatta* model induced by α-amanitin and lipopolysaccharide (H) (H and E stain, × 200); I: Normal liver of *Macaca mulatta* for comparison; J: The surface view of the experimental liver on necropsy appeared red and yellow; K: The cut surface view of the experimental liver was uniformly yellow.

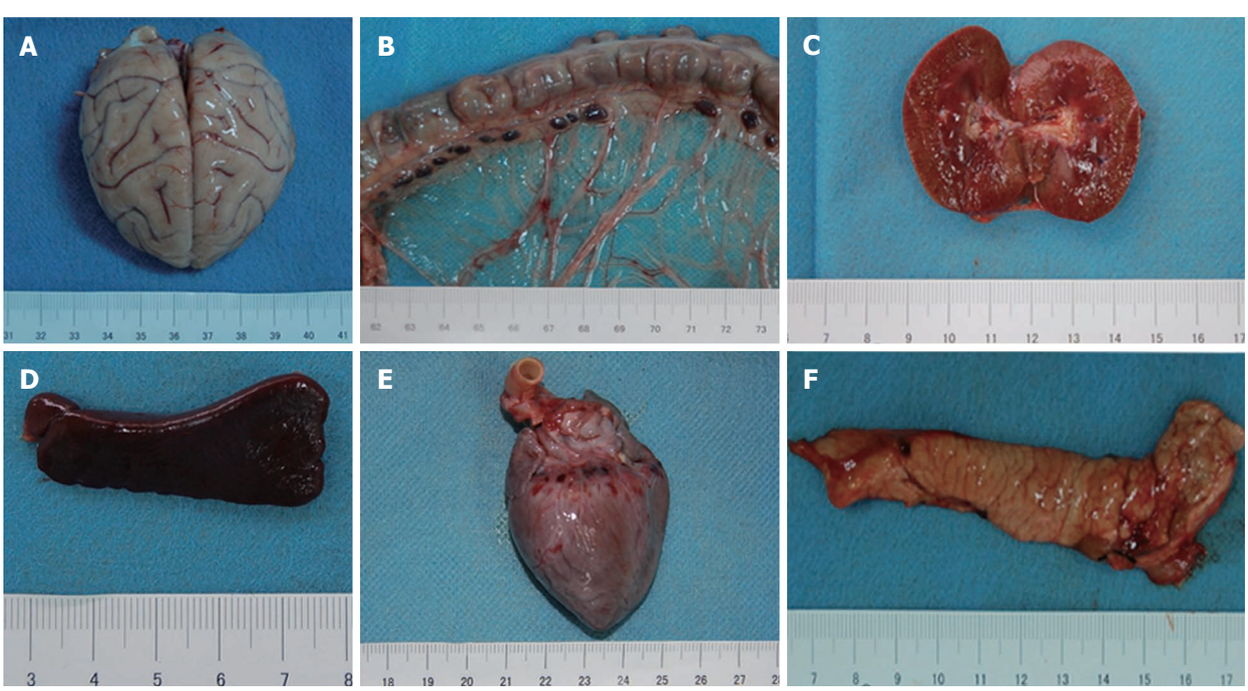

Figure 5 Other main organs on necropsy. A: The brain with cerebral edema; B: Mesenteric lymph nodes enlarged and beaded; Hyperemia of the kidney (C), spleen (D), heart (E), and pancreas (F) without obvious gross lesions.

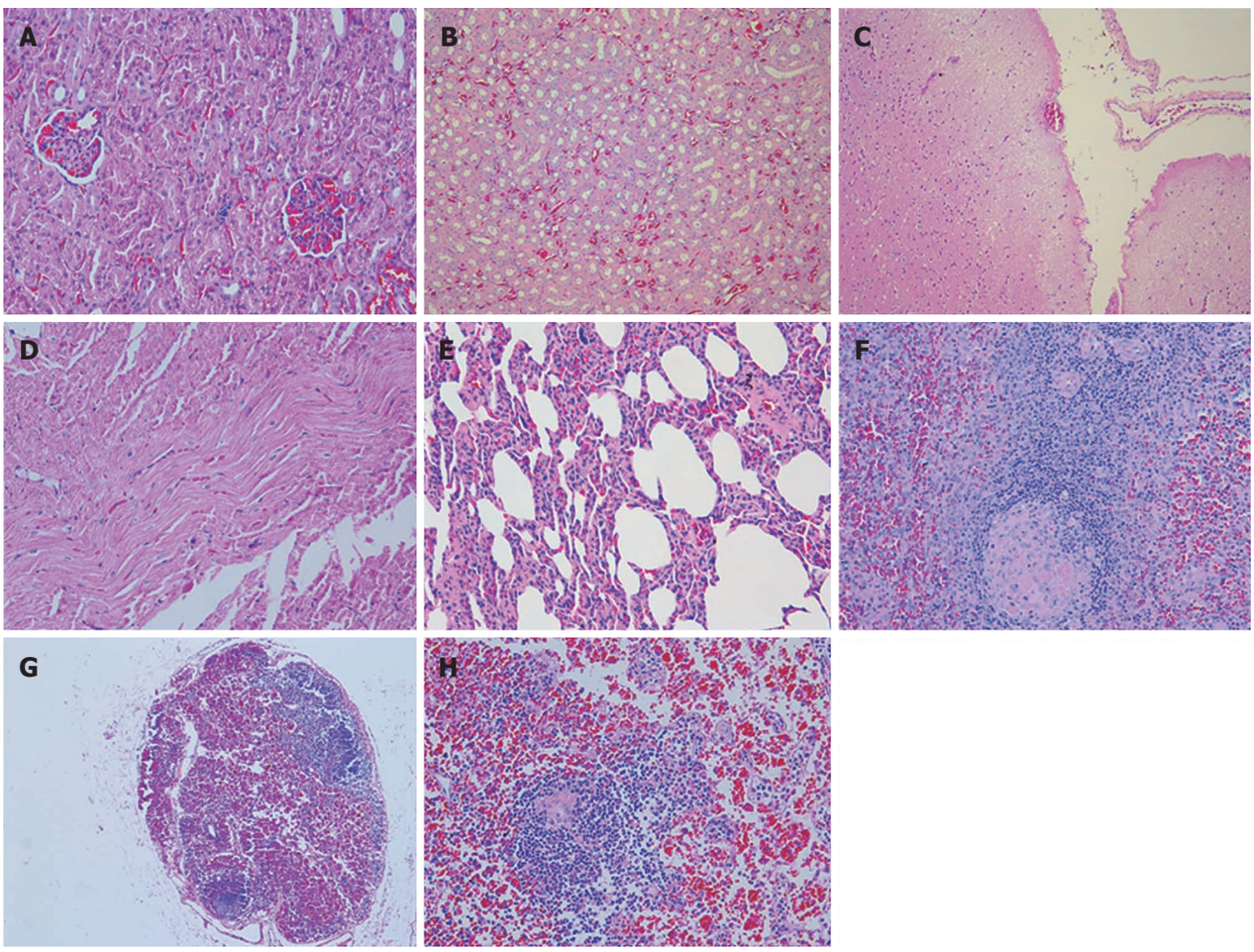

Figure 6 Histopathological changes of other organs. A: Ectasia and hyperemia of capillaries in the renal glomerulus [hematoxylin and eosin (H and E) stain, × 200]; B: Widespread hyperemia of capillaries in the renal stroma and cellular swelling in the tubular epithelial cells (H and E stain, × 100); C: Weak staining in the superficial layer of the brain suggest cerebral edema (H and E stain, × 100); D: Wave-like changes in the myocardium (H and E stain, × 200); E: Hyperemia of capillaries in the stroma and hyaline changes in the arterioles of the lung (H and E stain, × 200); F: Hyaline changes in the central artery of the spleen (H and E stain, × 200); G: Widespread reduction in lymphocytes and hemorrhage in the mesenteric lymph nodes (H and E stain, × 50); H: Hemorrhage and histiocyte proliferation in the mesenteric lymph nodes. Hyaline change appeared in the vessel wall of lymphoid follicles (H and E stain, × 200).

ical research. To the best of our knowledge, our *Macaca mulatta* model of FHF is the first large primate model in this field.

Methods used to induce the FHF models included surgical intervention and hepatotoxin administration. Total or partial hepatectomy and hepatic ischemic injury were widely used, but these relied on extensive surgical expertise and were influenced by individual variations in the animals. Hepatotoxins, which induce massive hepatic necrosis, are widely used in the establishment of a FHF model. Acetaminophen and galactosamine were the two most widely used hepatotoxins[17,19,22,26-31], and in some studies, carbon tetrachloride[32], thioacetamide[33,34], azoxymethane[35,36], concanavalin A[37] and amanita phalloides[38,39] were also employed. However, a lack of standardization, especially a lack of reproducibility, is the major disadvantage of models induced by acetaminophen[7,10]. The high costs restrict the application of galactosamine in large-scale models[40]. Amatoxin, a polypeptide extracted from a hypertoxic mushroom, has been used as a hepatotoxin in pigs with an endotoxin (LPS), and this model was reported to satisfy the criteria of near-universal mortality from highly reproducible hepatic failure, of mortality occurring in a defined time range, of damage specific to the liver, and of potential for recovery of the damaged liver[38,39]. In our preliminary experiment, LPS (2.25 μg/kg) and α-amanitin (0.225 mg/kg) were injected in a *Macaca mulatta* through a branch of the mesenteric vein by laparotomy at the dose converted from that used in pigs, which received LPS and α-amanitin at a dose of 1 μg/kg and 0.1 mg/kg, respectively[38,39]. However, the animal died within 8 h. We supposed that the direct and undiluted toxic effects to the liver might be the main cause of death and the surgery might promote the death of the *Macaca mulatta*. Thus, we reduced the dose of the two hepatotoxins to the level used in pigs, which was nearly half of our preliminary dose. We then improved the administration pathway by slow intraperitoneal infusion of the toxins diluted in 50 mL physiological saline instead of direct portal injection. Our results showed that the animals survived for at least 49 h and all the features of FHF were clearly presented. Most importantly, the

prolonged disease course ensures a wide therapeutic window. It is possible that intraperitoneal infusion of toxins could lead to severe peritonitis. Fortunately, we did not find any symptoms and signs of peritonitis, either in imaging examination or in necropsy.

Induced by α-amanitin and LPS, the hepatocytes presented severe steatosis first and massive necrosis at the end stage of FHF, which is similar to the case in humans suffering from acute severe viral hepatitis. Serum parameters, including hepatic enzymes, bilirubin, and coagulation activities, as well as serum ammonia, are often considered as the markers representing the level of damage of hepatocytes, since a biopsy is seldom carried out because of the restriction of invasive operation and bleeding. In fact, the hepatic injury during the course of FHF still needs direct confirmation. Our model offers an appropriate model to obtain real-time pathological changes in the hepatocytes, which makes it valuable for study of clinical features based on the pathological changes.

We also monitored the continuous alteration of the liver by imaging, and this is the first report on dynamic MRI of the liver during the course of FHF, either in patients or in an animal model. Combining the biopsy and the MRI, it is feasible to establish the diagnostic criteria of the damage level based on the MRI values, thus offering a non-invasive strategy to reveal the level of hepatic injury.

Hepatic encephalopathy is one of the most important clinical features and causes of death of FHF. Being consanguineous with humans, the *Macaca mulatta* presents similar behavior and consciousness to humans suffering from FHF. In addition, the brain volume and the similar gyrus outline make it suitable for MRI examination of the cerebral edema, ischemia or infarction. In our model, the progressing symptoms of listlessness, anorexia, grasping disability, mental indifference, drowsiness and coma are fully developed and thoroughly recorded. The behavior and conscious alterations, combined with the MRI and pathological changes, make it a valuable model for further investigation of the pathogenesis and pathophysiology of hepatic encephalopathy, which could not be reproduced in humans or in any other animals. As a noninvasive means of detection, MRI may be an appropriate strategy to monitor the progression of the hepatic encephalopathy. The first objective assessment of MR brain changes specific to hepatic encephalopathy was made in the early 1990s. It was noted that in patients with cirrhosis, the basal ganglia appeared hyperintense on T1-weighted MRI[41]. However, continuous data are still absent to reveal the correlation between the degree of encephalopathy and the MRI parameters during the course of FHF. With our model, it is feasible to obtain the dynamic imaging alterations during the whole course of hepatic encephalopathy.

It is necessary to rule out that animal death was induced by extrahepatic organ failure. In our experiment, the main extrahepatic organs were also evaluated. The increasing of CRE happened late, suggesting that the damage to the kidneys was a result of hepatorenal syndrome rather than direct toxin damage. The values of CK and LDH increased immediately after toxin administration, but no marked abnormality was found by echocardiogram and no myocardial necrosis was observed in histopathological examination. The cause of the increased CK and LDH still needs clarification. The lesion of the mesenteric lymph nodes might be associated with the toxins, which needs further study. We did not find any pathological evidences indicating cell injury of the lung and pancreas. Above all, we can conclude that the toxicity of intraperitoneal infusion of amatoxin and endotoxin is liver-specific and animal death is directly correlated with FHF and not extrahepatic organ failure.

We describe, for the first time, a *Macaca mulatta* model of fulminant hepatic failure, which satisfies all of the criteria for a large animal model of FHF[10,11]. With similar metabolic and physiological properties to humans, our primate animal model of FHF offers a valuable model to be used for investigation of the pathophysiology of FHF and for evaluation of potential medical therapies. Compared with other animals, primate models are more expensive, and the ethics of using such animals should be taken into consideration.

COMMENTS

Background

Fulminant hepatic failure (FHF) is an uncommon and challenging clinical disease characterized by sudden and severe hepatic injury and dysfunction, and it results in many different symptoms and complications with a high mortality. Previous reports have described different animal models of FHF induced by various methods, but all the established models did not entirely satisfy all the criteria.

Research frontiers

Animal models of FHF are urgently needed to fully investigate the pathogenesis, progression, diagnosis and treatment of this serious disease in the clinic. Recently, more studies have focused on large animal models and therapy for FHF. Novel therapeutic strategies including hepatocyte transplantation, stem cell transplantation, tissue engineered liver and BAL support systems are under investigation. Also, various animal models of FHF have been created with different methods, including models using rodents, dogs and pigs. However, differences in metabolic and physiological properties in these distantly relative species restricted the application of the results to humans. No primate animal model with FHF has been created before.

Innovations and breakthroughs

In this article, the authors describe, for the first time, a *Macaca mulatta* model of FHF, which satisfies all of the criteria for the large animal model of FHF. It is important that with the model, it is feasible to obtain dynamic imaging and pathologic alterations, and this is the first report about dynamic MRI of the liver during the course of FHF, either in patients or in animal models. Though lipopolysaccharide and α-amanitin were reported to induce a pig model of FHF, the main extrahepatic organs were not evaluated. In this article, the authors improved the administration pathway and evaluated the main extrahepatic organs.

Applications

The primate animal model of FHF offers a valuable model to be used for investigation of the mechanism of FHF and for evaluation of potential medical therapies.

Terminology

Macaca Mulatta: A species of the genus *MACACA* inhabiting India, China, and other parts of Asia. The species is used extensively in biomedical research and adapts very well to living with humans.

Peer review

The authors show an appropriate large primate model of fulminant hepatic failure. They described the rigid data of hepatic failure by biochemical results, imaging, and pathological changes. The primate animal model of fulminant hepatic failure has clinical benefit because of similar metabolic and physiological properties to human. This paper is an interesting and instructive manuscript. It is well written.

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BRIEF ARTICLE

Chronic fatigue is associated with increased disease-related worries and concerns in inflammatory bowel disease

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Abstract

AIM: To investigate the impact of chronic fatigue on disease-related worries in inflammatory bowel disease (IBD) and the potential multicolinearity between subjective questionnaires.

METHODS: Patients in remission or with mild-to-moderate disease activity completed the fatigue questionnaire (FQ), the rating form of IBD patient concerns (RFIPC), the Short-Form 36 (SF-36), and IBD questionnaire (N-IBDQ). In addition, clinical and epidemiological data were obtained.

RESULTS: In total, 140 patients were included; of which 92 were diagnosed with ulcerative colitis and 48 with Crohn's disease. The mean age of patients with chronic fatigue was 44.2 years (SD = 15.8) and for non-fatigued patients was 44.7 years (SD = 16.0). Chronic fatigued patients had clinically significantly increased levels of disease-related worries, as measured by Cohen's *d* effect size. Worries about having an ostomy bag, loss of bowel control, and energy levels were most prominent in both chronic fatigued and non-chronic fatigued IBD patients. Variance inflation factor (VIF) and tolerance indicated that there were no problematic multicolinearity among the FQ, RFIPC, SF-36 and N-IBDQ responses (VIF < 5 and tolerance > 2).

CONCLUSION: Chronic fatigue is associated with increased levels of disease-related worries and concerns in IBD. Increased levels of worries were also associated with impaired health-related quality of life.

Key words: Fatigue; Worries; Health-related quality of life; Patient reported outcome; Inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). Both UC and CD are chronic, recurrent diseases of the gastrointestinal tract, with symptoms that include abdominal pain, frequent bowel movements, and rectal bleeding[1,2]. Traditionally, these diseases have been defined as abnormalities in the structure and function of both organs and tissues[3]. However, given that IBD is associated with impaired health-related quality of life (HRQoL), increased fatigue, and increased worrying regarding the potential consequences of the disease[4-7], this definition seems too restrictive. The concept of illness might therefore be broader, embodying the subjective experiences of reduced health and bodily function[3].

Living with chronic illness can be challenging, and individual responses to these challenges vary widely[8]. Several HRQoL studies have found that many patients with IBD tend to do well[9]. However, there are subgroups of patients - such as patients with more severe disease or anxiety - who seem to be particularly affected[4,5,9]. It was recently reported that IBD patients with chronic fatigue (CF) (defined as an elevated fatigue level of a duration longer than six months) had clinically significant reductions in HRQoL, compared to patients without CF[10]. Thus, an important aspect of measuring subjective health is to identify patients who are most severely affected.

Both generic (i.e., irrespective of illness/condition) and disease-specific HRQoL questionnaires have been developed[11-13]. In addition, questionnaires such as the rating form of IBD patient concerns (RFIPC) and the fatigue questionnaire (FQ) are used to quantify other aspects of living with chronic illness[7,14]. We hypothesised that measuring aspects such as worries/concerns (RFIPC) and fatigue (FQ) might help us to identify subgroups of patients that tend to have a poorer outcome. Potentially, however, there might be areas of overlap between the various questionnaires, which is often referred to as multicolinearity. Multicolinearity refers to a situation wherein predictor variables in a regression model are strongly correlated and, consequently, that variables included in the model are closely related.

The primary aim of this study was to examine the impact of chronic fatigue on disease-related worries in IBD. In addition, we wanted to investigate whether the potential association between worries, fatigue and HRQoL in IBD exhibit multicollinearity.

MATERIALS AND METHODS

Subjects

Patients, who were over the age of 18 years, had IBD that was previously verified clinically, endoscopically, or histologically, and who were either in remission or with mild-to-moderate disease activity [defined as Simple Clinical Colitis Activity Index (SCCAI)[15] or Simple Crohn's Disease Activity Index (SCDAI)[16] score of less than 10], were eligible for inclusion in this study. Patients were excluded if they had cognitive impairment, were deemed unlikely to comply with the study procedures, or if they participated in another study. Participants were consecutively recruited from three outpatient clinics in South-Eastern Norway (the counties of Østfold and Hedmark) during routine follow-up visits. At each of the centre, a senior gastroenterologist was in charge of the study protocol. The inclusion period was from August 23, 2005 to January 29, 2007.

Clinical and sociodemographic data

Sociodemographic variables were gathered by interview, and data regarding clinical status and symptoms were obtained from laboratory tests, medical records and disease activity indices (SCCAI/SCDAI)[15,16]. In addition, we asked the patients to complete a symptom-based questionnaire that graded their self-perceived IBD symptoms during the previous 14 d, using the following categories; no symptoms, mild symptoms (did not interfere with everyday activities), moderate symptoms (interfered with everyday activities and may have resulted in sick leave), and severe symptoms (unable to perform everyday activities, on sick leave, or hospitalized)[5].

Each patient's phenotype was classified according to the Vienna Classification for CD patients, as the Montreal Classification did not exist when the study protocol was designed. The UC patients were classified into three subgroups: proctitis, left-sided colitis (with inflammation up to the splenic flexure), and extensive colitis (with inflammation beyond the splenic flexure).

The information regarding fatigue was collected with the FQ[14], the generic HRQoL with the Short-Form 36 (SF-36)[11], the disease specific HRQoL with the Norwegian version of the IBD questionnaire (N-IBDQ)[17], and disease-related worries and concerns with the RFIPC[7] (Table 1). The questionnaires were self-administered by the patients at the various centres, following a standardized procedure, which allowed the patients to fill out the questionnaires in the peace and quiet of the hospital's outpatient clinic.

Questionnaires

The RFIPC: The RFIPC is a disease-specific questionnaire that was developed by Drossman *et al*[7]. This questionnaire rates various important worries and concerns that are raised by IBD patients. The questionnaire consists of the 25 most frequently reported concerns reported by IBD patients, with every item framed in the same style: "Because of your condition, how concerned are you with…?" The responses were scored on a 100-mm horizontal visual analog scale. A score of 0-mm represents no worries/concerns, and a score of 100-mm represents the highest possible worries and concerns. The mean scores of all 25 items yields the "sum score". The RFIPC has been translated into Norwegian and validated (Jelsness-Jørgensen LP, Moum B, Bernklev T. Worries and concerns among inflammatory bowel disease patients followed prospectively over one year. Submitted:

Table 1 Main characteristics of the questionnaires used in this study

Questionnaire	Number of items and dimensions	Main characteristics
SF-36 Ware *et al*[11]	36 items, divided into 8 dimensions	Measure generic HRQoL Scale scores from 0-100, with higher scores indicating better HRQoL
N-IBDQ Bernlev *et al*[17]	32 items, divided into 5 dimensions	Measure disease-specific HRQoL Scale scores from 32-224, with higher scores indicating better HRQoL
FQ Chalder *et al*[14] Jelsness-Jørgensen *et al*[10]	11 items, divided into 2 dimensions The FQ contains 2 items asking about duration and extent of fatigue symptoms	Measure both physical and mental fatigue Scale scores from 0-33 with higher scores indicating higher levels of fatigue In addition scored as a dichotomized scale where original scores 0 + 1 = 0 and 2 + 3 = 1 Chronic fatigue defined as a score of ≥ 4 on the dichotomized scale and duration of fatigue symptoms ≥ 6 mo
RFIPC Drossman *et al*[7]	25 items, divided into 6 dimensions	Measure the 25 most frequently reported disease-related worries/concerns by IBD patients

IBD: Inflammatory bowel disease; SF-36: Short-Form 36; N-IBDQ: IBD questionnaire (Norwegian version); FQ: Fatigue questionnaire; RFIPC: Rating form of IBD patient concerns; HRQoL: Health-related quality of life.

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Factor analysis of the Norwegian version of the RFIPC yielded six factors. F1: Impact of disease (e.g., financial difficulties/energy/loss of bowel control); F2: Expectancy (e.g., developing cancer/dying early); F3: Treatment (e.g., effects of medication); F4: Intimacy (e.g., ability to perform sexually); F5: Stigma (e.g., feeling dirty/smelly); and F6: Complications (e.g., undergoing surgery/ostomy bag placement).

FQ: The FQ was developed by Chalder *et al*[14] and consists of 11 items that are divided into two main dimensions: physical fatigue (PF), which contains seven items, and mental fatigue (MF), which contains four items. The available responses included four options: 0 = better than usual, 1 = no more than usual, 2 = worse than usual, and 3 = much worse than usual. A higher score reflects a higher level of fatigue. Combining the scores of PF and MF yields the total fatigue score, with a maximum possible scale score of 33. The scale scores of the FQ were also scored on a dichotomized scale (0 = better than usual and no more than usual; 1 = worse than usual and much worse than usual). In addition to measuring episodic fatigue (irrespective of the duration of symptoms), the FQ contains two questions regarding the duration and extent of fatigue symptoms. Based on the results of the original validation study, the Norwegian validation study, and general consensus[14,18,19], CF was defined as dichotomized scores ≥ 4 and duration > 6 mo. The FQ was recently validated for use in IBD[6].

SF-36: The SF-36 is a generic, self-administered questionnaire containing 36 questions[11] that are divided into eight multi-item scales consisting of: physical functioning, role limitations due to physical problems, bodily pain, general health, vitality, social functioning, role limitations due to emotional problems, and mental health (MH). For each question, the raw score was coded and transformed into a scale from 0 to 100, with 0 and 100 representing the lowest level and highest level of function, respectively. The SF-36 has been validated by others[20]. Missing data were treated following published recommendations: if data for half of the items within a scale or fewer were missing, they were replaced by the mean value of the respondent's completed items in the same scale in accordance with the SF-36 scoring algorithms[21].

N-IBDQ: The IBDQ is a disease-specific questionnaire that was developed by Irvine[12,13]. The original version consists of 32 items divided into four dimensions: bowel symptoms (e.g., loose stools or abdominal pain), systemic symptoms (e.g., fatigue or altered sleep patterns), social function (e.g., work attendance) and emotional function (e.g., anger or depression). The Norwegian validation study (N-IBDQ) revealed a five-dimensional structure: emotional function-1 (fatigue, energy), bowel function-1 (stool consistency and pattern), bowel funtion-2 (bowel pain and discomfort), social function (work attendance, cancelling social events) and emotional function-2 (worries)[17]. All of the responses were scored on a 7-point Likert Scale, with a score of 7 representing no problems and a score of 1 representing severe problems. This gives a possible score range of 32-224, with a higher score reflecting improved HRQoL[12,13,17].

Statistical analysis

To assess the characteristics of the patients, we used descriptive analyses and frequencies. A student's *t* test was used to evaluate the differences in the distribution of epidemiological and clinical variables between the diagnostic groups.

To test potential associations between the RFIPC, FQ, N-IBDQ and SF-36 questionnaires, both bivariate correlation analysis with spearman's ρ and linear regression analysis were used. In addition, the latter analysis was used to explore potential multicollinearity. A variance inflation factor (VIF) greater than 5 and a reciprocal tolerance value of less than 0.20 were defined as indicative of colinearity in accordance with published recommendations[22,23]. We chose to analyse the RFIPC sum score, the FQ and N-IBDQ total score as dependents only, to reduce the number of dimensions tested.

Table 2 Main clinical/sociodemographic characteristics *n* (%)

	UC (*n* = 92)	CD (*n* = 48)
Age, (yr), mean ± SD	46.9 ± 5.8	40.0 ± 15.0
Age range, (yr)	20-82	19-69
Gender		
Female	43 (47)	36 (75)
Male	49 (53)	12 (25)
Disease duration, (yr), mean ± SD	8.5 ± 9.5	9.2 ± 9.6
Educational level		
Second level, first stage (lower)	16 (17.4)	5 (10.4)
Second level, second stage (medium)	42 (45.7)	22 (45.8)
Third level (university)	34 (37.0)	21 (43.8)
UC extension		
Proctitis	27 (29.3)	
Left-sided colitis	23 (25.0)	
Extensive colitis	42 (45.7)	
CD extension		
L1 terminal ileum		11 (22.9)
L2 colon		17 (35.4)
L3 ileocolon		18 (37.6)
L4 upper GI		2 (4.1)
Perceived IBD symptom score		
No symptoms	23 (25.0)	9 (18.8)
Mild symptoms	41 (44.6)	22 (45.8)
Moderate symptoms	25 (27.2)	13 (27.1)
Severe symptoms	3 (3.2)	4 (8.3)

UC: Ulcerative colitis; CD: Crohn's disease; IBD: Inflammatory bowel disease; GI: Gastrointestinal.

To control for and eliminate the effect of potential confounding factors that are known to influence RFIPC scores, age, gender, and educational level were entered as covariates through univariate analysis of variance. Perceived IBD symptoms were hypothesized to affect the RFIPC scores and we therefore additionally chose to correct for this factor as well. The effect size was calculated with Cohen's *d*[24]. Operational definitions of 0.2, 0.5 and 0.8 were categorized as small, medium and large effect sizes, respectively[24].

All tests were 2-sided with a 5% significance level. All statistics were performed using the Predictive Analytics Software, PASW, version 18.0 (IBM Corporation, Route 100 Somers, NY 10589).

Ethical considerations

This study was performed in accordance with the principles of the Helsinki declaration and approval was obtained from the Regional Ethics committee and the Norwegian Data Inspectorate.

RESULTS

One hundred and forty-four patients who were diagnosed with either UC or CD gave their written informed consent for participation in the study. One patient was excluded due to severe disease activity at inclusion (SCDAI > 10), one patient withdrew from the study after a few weeks, and two patients were excluded from analysis due to incomplete responses to the questionnaires. A total of 140 patients provided complete data sets and were suitable for statistical analysis.

Epidemiological and clinical characteristics

The CD patients were significantly younger than the UC patients ($P = 0.014$). There were no significant differences in the perceived IBD symptoms or the duration of disease between the two diagnostic groups. Twenty of the 92 (22%) UC patients and 14 of the 48 (29%) CD patients were judged to have CF. There were no significant differences in age, gender, disease duration or educational level between CF and non-CF patients. However, the perceived IBD symptoms were more severe in CF than in non-CF patients ($P < 0.01$). The primary characteristics of the participants are presented in Table 2.

CF and RFIPC scores

CF was associated with significantly higher RFIPC scores in one of the six dimensions in CD and in five of the six dimensions in UC. In addition, the RFIPC sum score was significantly higher in UC patients with CF compared to those without CF (Table 2). Among the individual RFIPC items, the differences between UC patients with and without CF were most pronounced in worries regarding loss of bowel control, developing cancer, dying early, energy level, being a burden to others, having surgery, or an ostomy bag ($P < 0.01$ for all items). In addition, the scores for the following RFIPC items were significantly higher in CD patients with CF compared to those without CF: ability to achieve full potential, financial difficulties, and energy level ($P < 0.05$ for all items). When comparing RFIPC dimensional scores for UC patients with CF to CD patients with CF, the analysis revealed no statistical difference. Only one of the twenty-five individual RFIPC items (worries about pain and suffering) was significantly different between UC and CD patients with CF, with CD patients reporting more worries in this item ($P < 0.01$).

Effect size (Cohen's *d*) is a measure of the estimated magnitude of a relationship between two variables and is calculated by subtracting the mean dimensional RFIPC scores of CF patients from non-CF patients, then dividing by the common σ of both groups. The analysis revealed that the RFIPC factors that were statistically significant in CF *vs* non-CF patients - regardless of their IBD diagnosis - also produced a large Cohen's *d* ($d > 0.80$) (Table 3). The analysis further revealed that although they did not reach statistical significance, the numerical differences produced small-to- medium effect sizes. Among the CD group, the differences between CF and non-CF patients resulted in a medium effect size ($d > 0.50$) in the RFIPC sum score, whereas the effect size in the remaining five out of six dimensions were small ($d > 0.20$). Among the UC group, one out of the six RFIPC factors did not differ significantly between CF and non-CF patients, producing a small Cohen's *d* ($d > 0.20$).

After the mean dimensional RFIPC scores (Table 4)

Table 3 Cohen`s *d* effect size calculated from crude dimensional rating form of inflammatory bowel disease patient concerns scores and presence of chronic fatigue

RFIPC factors	CD no CF (*n* = 34)	CD CF (*n* = 14)	*P* value	Cohen`s *d*	UC no CF (*n* = 72)	UC CF (*n* = 20)	*P* value	Cohen`s *d*
Impact of disease	31.1 (20.6)	46.7 (17.7)	0.017	-0.81	25.6 (19.2)	45.7 (18.3)	< 0.001	-1.07
Expectancy	38.1 (30.5)	51.1 (23.1)	0.160	-0.48	32.6 (23.7)	55.3 (20.8)	< 0.001	-1.02
Treatment	27.5 (21.2)	38.0 (25.4)	0.146	-0.44	27.5 (22.0)	49.1 (22.5)	< 0.001	-0.97
Intimacy	20.3 (21.6)	28.3 (28.5)	0.299	-0.31	18.5 (18.9)	27.2 (20.1)	0.076	-0.44
Stigma	21.2 (24.3)	26.6 (20.2)	0.467	-0.24	17.3 (19.7)	35.0 (22.0)	0.001	-0.84
Complications	26.3 (23.6)	30.6 (18.6)	0.549	-0.20	21.2 (17.9)	35.7 (17.3)	0.002	-0.82
Sum score	29.2 (19.1)	39.6 (16.3)	0.082	-0.58	25.0 (15.9)	43.5 (14.5)	< 0.001	-1.21

Data are presented as mean (SD), calculated with independent samples *t* tests. RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; CF: Chronic fatigue. Cohen`s *d* effect size: small, *d* = 0.2; medium, *d* = 0.5; large, *d* = 0.8.

Table 4 Univariate analysis of mean rating form of inflammatory bowel disease patient concerns scores in non-chronic fatigue and chronic fatigue patients

	Mean RFIPC adjusted for age, gender and education		Mean RFIPC adjusted for age, gender, education and IBD symptoms	
	No CF mean	CF mean	No CF mean	CF mean
CD (*n* = 48)	(*n* = 34)	(*n* = 14)	(*n* = 34)	(*n* = 14)
Impact of disease	30.7	47.6[a]	32.2	44.0[a]
Expectancy	37.3	53.0	37.8	52.0
Treatment	27.5	38.0	27.2	39.0
Intimacy	19.7	30.0	20.6	27.8
Stigma	20.9	27.3	20.8	27.6
Complications	26.4	30.3	25.9	31.8
Sum score	28.8	40.3	29.4	39.0
UC (*n* = 92)	(*n* = 72)	(*n* = 20)	(*n* = 72)	(*n* = 20)
Impact of disease	25.7	45.3[c]	26.3	43.1[c]
Expectancy	33.0	53.8[b]	33.4	52.4[c]
Treatment	27.9	47.8[c]	28.5	45.6[c]
Intimacy	18.7	26.7	19.0	25.6[a]
Stigma	17.5	34.3[b]	17.9	33.1[c]
Complications	21.4	35.0[b]	21.7	34.0[b]
Sum score	25.2	42.8[c]	25.6	41.2[c]

RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; CF: Chronic fatigue; IBD: Inflammatory bowel disease. Significance levels are between patients reporting CF or not: [a]$P \leqslant 0.05$, [b]$P \leqslant 0.01$, [c]$P \leqslant 0.001$.

were adjusted for the covariates age, gender and education, statistically significant differences were reproduced in the same RFIPC factors as in the raw, unadjusted analysis for both the UC and CD patients. Furthermore, when controlling for the perceived IBD symptom score, the significance level increased in two of the six factors (expectancy/stigma) for the UC group. One RFIPC factor (intimacy) in the UC group changed from non-significant to significant. Among the CD patients, controlling for perceived IBD symptoms had only a minor effect.

The correlation between RFIPC sum score, total fatigue, N-IBDQ total score and subdimensions of the SF-36 questionnaire

In both the UC and CD patient groups, increased RFIPC sum scores were associated with higher fatigue levels (measured as total fatigue) and reduced HRQoL (Table 5).

Table 5 Correlation (Spearman's ρ) between rating form of inflammatory bowel disease patient concerns sum, fatigue questionnaire sum, Norwegian inflammatory bowel disease questionnaire total and Short Form 36 dimensions

	CD (*n* = 48) RFIPC sum score	UC (*n* = 92) RFIPC sum score
FQ		
TF	0.36	0.49
N-IBDQ		
IBDQ Total	-0.53	-0.51
SF-36		
PF	-0.34	-0.40
RP	-0.28	-0.48
BP	-0.22	-0.44
GH	-0.49	-0.43
VT	-0.32	-0.48
SF	-0.40	-0.57
RE	-0.12	-0.49
MH	-0.31	-0.50

RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; FQ: Fatigue questionnaire; TF: Total fatigue; N-IBDQ: Norwegian inflammatory bowel disease questionnaire; SF-36: Short Form 36; PF: Physical functioning; RP: Role physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role emotional; MH: Mental health.

The latter showed a negative Spearman's ρ both for N-IBDQ and the SF-36 subdimensions. In general, these associations were stronger for the UC group than for the CD group.

Multicolinearity

Testing for multicollinearity revealed satisfactory values of both the VIF and tolerance (Table 6). The VIF was below the limit of 5 and the tolerance above the crucial threshold of 0.2 for both the UC and CD group. In the CD group, the ranges for the VIF and tolerance were 1.6-4.9 and 0.21-0.61, respectively. In the UC group, the ranges of the VIF and tolerance were 1.9-4.1 and 0.24-0.54, respectively.

DISCUSSION

The ramifications of IBD on patients subjective health has been thoroughly studied, identifying both a sub-

Table 6 Linear regression analysis of the rating form of inflammatory bowel disease patient concerns sum score, Norwegian inflammatory bowel disease questionnaire total score, total fatigue, chronic fatigue, Short Form 36 subdimensions and calculation of multicolinearity

	Dependent	Independent	ß	*P* value	Tolerance	VIF
UC	RFIPC sum	N-IBDQ total	-0.70	< 0.001	0.24	4.1
	N-IBDQ total	RFIPC sum	0.29	< 0.001	0.58	1.7
		SF-36 BP	0.14	0.032	0.57	1.7
		SF-36 VT	0.17	0.019	0.50	2.0
		SF-36 SF	0.29	< 0.001	0.39	2.6
	TF	SF-36 VT	-0.25	0.031	0.49	2.0
	CF	RFIPC sum	0.34	0.018	0.46	2.2
CD	RFIPC sum	N-IBDQ total	-0.60	0.047	0.21	4.9
	N-IBDQ total	RFIPC sum	-0.17	0.047	0.71	1.4
		SF-36 SF	0.35	0.004	0.39	2.6
		SF-36 PF	0.22	0.033	0.49	2.0
		TF	-0.31	0.015	0.34	2.9

RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; TF: Total fatigue; CF: Chronic fatigue; N-IBDQ: Norwegian inflammatory bowel disease questionnaire; SF-36: Short Form 36; PF: Physical functioning; BP: Bodily pain; VT: Vitality; SF: Social functioning; VIF: Variance inflation factor.

group of patients with a worsening in HRQoL scores and other subgroups of patients with HRQoL scores that are comparable to the general population[4,5,9,15]. This division of patient responses to IBD can partly be explained by demographic differences and differences in clinical variables[4,5,9,15]. Recently, CF was reported to be at least twice as prevalent in IBD as in the general population, and CF has been reported to lead to clinically significant reductions in HRQoL[6,10].

In the present study, we hypothesized that chronic fatigue might influence the level of disease-related concerns in IBD patients, and we found significant differences in the RFIPC scores between UC patients with CF and UC patients without CF. Although only one factor was significantly higher for CD patients with CF compared to CD patients without CF, there was a tendency of elevated scores in all RFIPC factors. In addition, we calculated effect sizes according to Cohen[24], because statistical significant differences need not be of clinical importance and insignificant differences might be. As expected, the effect sizes were more pronounced in UC patients than in CD patients. The five RFIPC factors that were not significant in CD were, however, within Cohen's limits of small-to-medium effect sizes[24]. This finding indicates that there are clinically important differences between IBD patients with CF and IBD patients without CF among all of the RFIPC scales.

IBD is often characterized by periods of remission and exacerbation; therefore, potentially negative consequences of the disease may become the primary focus in daily living[1,2,5]. This has also been reported by Drossman[7]. Worries and concerns are aspects of subjective health that can lead to decreased well-being[7]. These worries may potentially be linked to situations in which the patient's expectations regarding physical and mental functioning in "normal life" do not match their experienced reality[8].

Within the published literature, there appears to be a pattern regarding which of the individual RFIPC items are rated by IBD patients as being most important[7,25-27]. Our results concur with previous studies in this regard. However, the presence of chronic fatigue seems to further increase worries in IBD patients. To our knowledge, this association has not been previously reported for IBD. Among CD patients, we found that CF was associated with increased worries regarding pain and suffering. Research has highlighted the negative association between body pain and CD[5] and recently it was reported that chronic fatigue further decreased body pain scores[10]. In addition, patients with CD and CF seem to negatively relate their perceived energy capacity with their ability to achieve full potential and experience financial difficulties. The latter may be related to their ability to work and provide a household income. Indeed, studies have found that patients who are on sick-leave because of their IBD have a significant deterioration in HRQoL[28].

The nature of worry is not clear; however, it is most often prospective and predominated by negative thinking[29]. In IBD patients, these processes are linked to potentially negative events that may or may not occur in the future, including requiring surgery or an ostomy bag[3,7,25-27]. IBD patients are reported to believe that stress influences the course of the disease[30]. However, this potential link between psychological stress and inflammatory exacerbations is the subject of debate[31-33]. Addressing the worries and concerns of patients might therefore have a potentially positive influence on both fatigue and HRQoL; on the other hand, Borgaonkar *et al*[34] found that providing disease-related information to IBD patients worsens their short-term HRQoL.

In the present study, we found that increased levels of worrying were associated with both increased fatigue levels and reduced HRQoL. When investigating the potential association between worries, fatigue, and HRQoL in IBD patients, it is natural to wonder which is the cause and which is the effect. Is increased worrying a result of chronic fatigue or vice versa? Does impaired HRQoL increase worries and fatigue, or is HRQoL exaggerated by worries? The cross-sectional design of our study makes it difficult to reach any final conclusions. Our hypothesis, which we regard as plausible, is that both reduced HRQoL and increased fatigue are secondary effects of increased disease-related worries and concerns. This theory has also been proposed by others[30]. Potentially, elevated levels of disease-related concerns may increase energy loss and thus manifest as increased fatigue and reduced HRQoL[35].

The RFIPC was developed just a few years after the IBDQ, which may explain why the questionnaire has received much less attention in the literature[7,12,36,37]. As shown by our study, employing a set of various questionnaires that measure subjective health increases the

likelihood of detecting subgroups of IBD patients who are at risk of coping less successfully[8]. Naturally, there is the potential risk that subdimensions of different questionnaires are in fact operationalizations of the same phenomena[22,23]. However, both the VIF and tolerance were within acceptable limits for both UC and CD patients, indicating that the questionnaires seem to measure different aspects of health perception. Given these results, we would argue that patient-reported outcome, rather than HRQoL, seems to be a more adequate definition in cases were one is interested in a wide variety of outcomes (e.g., worries, concerns, fatigue, HRQoL).

A limitation of the present study is that we did not include a specific questionnaire for measuring depression. This precludes the possibility of adjusting for depression as a potentially confounding variable for fatigue. Several reports have indicated a connection and overlap between fatigue and depression[7,19,38]. However, the MH dimension of the SF-36 questionnaire indicated that the patients in our study did not differ from the general population in this respect. Consequently, depression does not appear to be a particular problem in this patient population. Moreover, in the CD patient group there may be type 2 statistical errors, as we were not able to obtain the strong associations between levels of worries and fatigue, as was shown for UC patients.

In conclusion, we report that chronic fatigue is associated with clinically significantly increased levels of disease-related worries in both UC and CD patients. This study provides additional information to the complex nature of understanding how IBD patients perceive their own subjective health.

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COMMENTS

Background

Fatigue is reported to be a prevalent symptom in chronic diseases. In inflammatory bowel disease (IBD), however, there has been a lack of studies with fatigue as the primary endpoint. Recent publications have revealed that the amount of patients reporting long-lasting, chronic fatigue is two to three times elevated in patients with IBD compared to the background population.

Research frontiers

Chronic fatigue has been reported to significantly reduce IBD patients' health-related quality of life. In this study, the authors demonstrate that chronic fatigue is a potential important contributor to increased disease-related worries and concerns.

Innovations and breakthroughs

Recent studies have highlighted the importance of fatigue as a subjective symptom in IBD. This is the first study to report that chronic, long-lasting fatigue symptoms is associated with increased levels of disease-related worries.

Applications

By adding further complexity to the understanding of subjective health experiences in IBD, this study might help clinicians to detect patients at risk of less successful coping, potentially enhancing the patient's quality of life, and also their course of disease.

Terminology

Chronic fatigue refers to fatigue symptoms of some intensity, which have a long-lasting duration of six months or more.

Peer review

This is a very interesting and novel original contribution analyzing the problem of chronic fatigue and its relationship with worries associated with IBD. The study is interesting, seems well done and well reported. It adds a valuable bit of information to the IBD literature.

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BRIEF ARTICLE

Pneumatosis cystoides intestinalis: A single center experience

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Abstract

AIM: To share our experience of the management and outcomes of patients with pneumatosis cystoides intestinalis (PCI).

METHODS: The charts of seven patients who underwent surgery for PCI between 2001 and 2009 were reviewed retrospectively. Clinical features, diagnoses and surgical interventions of patients with PCI are discussed.

RESULTS: Seven patients with PCI (3 males, 4 females; mean age, 50 ± 16.1 years; range, 29-74 years) were analyzed. In three of the patients, abdominal pain was the only complaint, whereas additional vomiting and/or constipation occurred in four. Leukocytosis was detected in four patients, whereas it was within normal limits in three. Subdiaphragmatic free air was observed radiologically in four patients but not in three. Six of the patients underwent an applied laparotomy, whereas one underwent an applied explorative laparoscopy. PCI localized to the small intestine only was detected in four patients, whereas it was localized to the small intestine and the colon in three. Three patients underwent a partial small intestine resection and four did not after PCI was diagnosed. Five patients were diagnosed with secondary PCI and two with primary PCI when the surgical findings and medical history were assessed together. Gastric atony developed in one case only, as a complication during a postoperative follow-up of 5-14 d.

CONCLUSION: Although rare, PCI should be considered in the differential diagnosis of acute abdomen. Diagnostic laparoscopy and preoperative radiological tests, including computed tomography, play an important role in confirming the diagnosis.

Key words: Pneumatosis cystoides intestinalis; Peritoneal free air; Radiological tools; Diagnosis

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INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a relatively uncommon condition, characterized by the presence of multiple gas-filled cysts within the wall of the gastrointestinal tract[1-12]. The term "pneumatosis intestinalis" was first used by Duo Vernoi while observing autopsy specimens in 1730. The entity defined by Duo Vernoi is what we now know as primary PCI. The term "second-

ary PCI" was termed by Koss in 1952, who analyzed 213 pathological specimens and attributed 85% of the cases to a secondary disease[1,2].

One of the pathognomonic features of PCI is pneumoperitoneum without peritoneal irritation as a result of a cyst rupture. In contrast, air retention leading to acute abdominal findings may be seen in some cases[3].

PCI is a radiological or exploratory entity, not a disease, and the underlying causes are numerous. PCI may develop either after a benign procedure, such as endoscopy, or from an unknown cause (primary or idiopathic). In some cases, a more serious disease, such as secondary necrotizing enterocolitis, may be the cause. No clear consensus has yet been established, although many mechanical, bacterial, and pulmonary hypotheses have been proposed regarding the etiopathogenesis of PCI[4]. PCI usually does not lead to clinical findings and may disappear spontaneously in cases in which the primary disease is treated. Steroids, an elemental diet, hyperbaric oxygen, antibiotics, and surgery have been used as treatments. In this study, we describe seven PCI cases, which were diagnosed and treated at our clinic.

MATERIALS AND METHODS

Seven patients were admitted to Firat University Faculty of Medicine, Department of Surgery, Emergency Unit, between January 2001 and August 2009. Their medical records were evaluated retrospectively to obtain follow-up and clinical data, including age, sex, initial complaints, medical histories, white blood cell, abdominal and thoracic radiography, intraoperative findings, surgical intervention, duration of hospital stay, complications and follow-up time (Table 1). Preoperative tests were performed, including routine biochemistry and thoracic and abdominal radiography. Five of the patients had findings consistent with an acute abdomen and were operated on. Contrast-enhanced abdominal computed tomography (CT) was performed in one case due to vague abdominal findings. The CT findings were consistent with a rectal perforation. Abdominal ultrasonography (USG) was also used in one patient who had marked tenderness in the right upper quadrant. All patients operated on underwent emergent surgery, and all patients were diagnosed with PCI intraoperatively. Surgical team consensus was used to determine which patients would be resected after a laparotomy and/or laparoscopic exploration. The affected segment was resected in cases with suspicion of bowel perforation and ischemia, whereas no additional surgical intervention was performed in cases in which only PCI was detected. The primary or secondary nature of the PCI was determined by considering the medical history and preoperative findings. Cases with no underlying predisposing disease were considered primary or idiopathic PCI, whereas those accompanying some disease, such as appendicitis, Crohn's disease, pyloric stenosis, necrotizing enterocolitis, peptic ulcers, cystic fibrosis, or chronic obstructive lung disease, were regarded as secondary PCI. Follow-up time was determined from the time of surgery to the last visit to our outpatient clinic.

RESULTS

Data for seven patients with PCI (3 males, 4 females; age, 50 ± 16.1 years (mean ± SD); range, 29-74 years) were analyzed retrospectively. Of the patients who presented at the Emergency Unit, three had severe abdominal pain, two had abdominal pain and vomiting, and two had abdominal pain, vomiting, and constipation. Liver and renal function tests as well as electrolyte values were normal in all patients, while marked leukocytosis was detected in four. Exam findings in six of the patients were consistent with acute peritonitis, whereas no findings other than minimal tenderness were noted in one patient (female, aged 29 years). Subdiaphragmatic free air was detected on plain thoracic and abdominal radiographs in four patients. Abdominal USG used in one patient (female, aged 74 years), who had marked tenderness in the right upper quadrant, revealed cholecystitis together with an image consistent with a stone in the lower tip of the choledoch. An abdominal CT of a 29-year-old female patient with vague findings revealed free air extending into the retroperitoneum, indicating a rectal perforation. This patient was diagnosed with acute abdomen and was scheduled for urgent surgery. A PCI diagnosis was established in seven patients after a laparotomy in six and a laparoscopic exploration in one. An appearance consistent with PCI was observed in the small intestine of four patients and in the small intestine and colon of three (Figure 1A). A small intestinal perforation was observed in only one (female, aged 34 years) of these cases (Figure 1B). PCI was detected incidentally in a 74-year-old female patient who was scheduled for bile duct surgery (cholecystectomy and choledoch exploration only). Three patients underwent a partial small intestinal resection and anastomosis, while four had no additional surgical procedures after PCI was diagnosed. All patients were given 3 L/min oxygen during the first 3 postoperative days. During the 5-14 d clinical follow-up, a 48-year-old male developed gastric atony, whereas the remaining six patients were discharged with no complications. No additional complications were observed in any of the patients during the 15 ± 5.4 mo (range, 8-23 mo) follow-up. After considering the surgical findings and medical and surgical histories, five of these patients had secondary PCI and two had primary PCI.

DISCUSSION

PCI is a rare condition characterized by multilocular gas-filled cysts localized in the submucosa and subserosa of the gastrointestinal tract[5,13-17]. The term "pneumatosis intestinalis" was first used by Duo Vernoi during postmortem observations. A PCI diagnosis in surviving patients was first established by Hahn in 1899. A PCI diagnosis *via* preoperative radiological findings was first

Table 1 Demographic and clinical characteristics of the seven patients with pneumatosis cystoides intestinalis

No.	Age	Sex	Complaint	Medical history	WBC	Radiologic tools	Loc.	Etiology	Surgical intervention	Length of hospital stay (d)	Postoperative complication	Follow-up (mo)
1	29	F	AP + V	Endoscopy	11.1	X-ray, CT[4]	SB	Secondary	Ileal resection + anastomosis	7	No	14
2	48	M	AP + V + C[2]	Peptic ulcer perforation	NR	X-ray	SB	Secondary	Ileal resection + anastomosis	14	Gastric atony	21
3	71	M	AP + V + C[2]	CLL (CT[3])	35	X-ray	SB	Secondary	Laparatomy	5	No	8
4	74	F	AP	Normal	NR	X-ray, US	SB	Secondary	Cholecystectomy + choledocotomy + drainage	11	No	23
5	53	F	AP	Colonoscopy	NR	X-ray	SB + C[1]	Secondary	Laparatomy	8	No	18
6	34	F	AP + V	Normal	23	X-ray	SB + C[1]	Primary	Ileal resection + anastomosis	10	No	12
7	41	M	AP	Normal	16	X-ray	SB + C[1]	Primary	Laparoscopic exploration	7	No	9

WBC: White blood cell; SB: Small bowel; [1]C: Colon; AP: Abdominal pain; V: Vomiting; [2]C: Constipation; CLL: Chronic lymphocytic leukemia; [3]CT: Chemotherapy; NR: Normal range; [4]CT: Computed tomography; US: Ultrasonography.

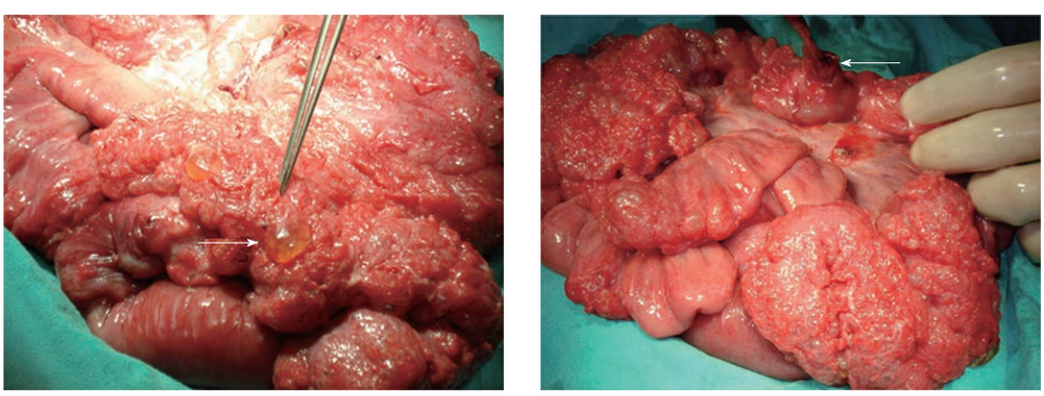

Figure 1 Intraoperative appearance of multiple air sacs in the small intestine. A: Multiple air sacs (white arrow); B: A perforation and multiple air sacs (white arrow).

described by Baumann-Schender in 1939. The condition originally described by Duo Vernoi is what we now consider primary PCI. The term "secondary PCI" was coined by Koss in 1952, who analyzed 213 pathological specimens and attributed 85% of the cases to a secondary disease[1-3].

Several hypotheses have been proposed regarding the development of PCI, although its pathogenesis is still controversial. Two main hypotheses regarding the fundamental pathogenesis of PCI are mechanical and bacterial[17]. The mechanical hypothesis postulates that PCI develops when defects in the mucosa, in combination with increased intraluminal pressure, allow gas to infiltrate the gastrointestinal (GI) tract wall. A subgroup of patients with severe pulmonary conditions may present with PCI arising from pulmonary causes, such as cough and rapid changes in intra-abdominal pressure. The bacterial hypothesis proposes that PCI develops when gas-producing bacteria gain entry into the GI tract wall and produce gas pockets. Much of the supporting evidence for these two hypotheses is derived from observational studies, and mechanical and bacterial mechanisms may occur simultaneously[4,6,7].

Although PCI may occur anywhere in the gastrointestinal tract, from the esophagus to the rectum, it is usually seen in the intestine. A previous study reported that 20%-51.6% of all PCI cases involve the small intestine, 36%-78% involve the colon, and 2%-22% include both the small intestine and colon. The small intestine was involved in 57.1% of the cases we presented here and 42.9% involved the small intestine and the colon[1,2,6,8].

PCI is not a disease but a clinical entity. The etiology can be classified by considering factors thought to play a role in its development. Based on this notion, PCI can be divided into primary and idiopathic (15%) or secondary (85%) type[9]. No identifiable underlying or predisposing factor is present in the primary or idiopathic type. However, numerous gastrointestinal diseases, including appendicitis, necrotizing enterocolitis, Crohn's disease, pyloric stenosis, ulcerative colitis, diverticular disease, necrotizing enterocolitis, gastroduodenal ulcer, and sigmoid volvulus, may accompany PCI as a secondary cause. PCI has also been reported as accompanying some non-gastrointestinal diseases, such as chronic obstructive pulmonary disease, collagen tissue diseases, acquired immune deficiency syndrome, and glucocorticoid use. PCI

cases secondary to surgical or endoscopic trauma have also been reported[10,11].

Lesions are usually localized to the left hemicolon or its mesentery or to the submucosal layer and are frequently characterized by segmentary involvement in the primary form of the disease. However, involvement is usually subserosal in the secondary form, and occurs in the stomach, small intestine, and right colon, usually in a generalized or segmented pattern[7].

The incidence of PCI is unknown, because it is usually asymptomatic. Symptoms, if any, are usually secondary to an underlying disease. Together with non-specific symptoms, such as abdominal discomfort, diarrhea, constipation, rectal bleeding, tenesmus, or loss of weight, severe complications, including volvulus, intestinal obstruction, tension pneumoperitoneum, bleeding, intussusception, and intestinal perforation may be seen in 3% of patients[18-23].

Radiological tools are important for diagnosing PCI. These include plain radiographs, USG, barium series, CT, CT-colonoscopy, magnetic resonance imaging and MRI-colonography, endoscopy, and colonoscopy[19,20]. X-ray is of great importance, because it is readily available in every emergency room. Cysts usually appear as radiolucent shadows, similar to a bunch of grapes, close to the intestinal lumen on radiographs. Free air underneath the diaphragm may be seen if these cysts perforate. An appearance of bulging into the lumen as a filling defect is seen on barium-colon radiographs[15,23]. Linear or spot-like hyperechoic images may be seen in the intestinal wall on USG. CT is the most useful method for diagnosing PCI and is important because it provides data on other abdominal pathologies. However, CT may not provide data on intestinal ischemia and necrosis[1,4,7,12]. The colonoscopic findings may be similar to multiple polyposis or collections of submucosal tumors, but subserous pneumatosis may go undetected[20]. A laparoscopic exploration is quite useful to confirm a PCI diagnosis, if the physical examination findings are suspicious, and particularly in cases that are not preoperatively diagnosed clearly using the above-mentioned radiological methods. Diagnostic laparoscopy provides the convenience of converting to open surgery as well as confirming the diagnosis.

When presence of such an entity is confirmed radiologically, gastroenterologic surgeons begin to feel annoyance. The answer to the question, "What should we do to these patients?" is correlated with the experience of each surgeon on that entity. The approach to a patient with PCI should be determined by evaluating the underlying causes and exam findings together. A specific treatment is not recommended in asymptomatic patients who are detected as having PCI radiologically and whose examination findings are negative. Conservative approaches, including nasogastric decompression, intestinal rest, antibiotic therapy and oxygen, are recommended for patients with positive examination findings and normal biochemical parameters who are confirmed radiologically to have no intestinal ischemia or perforation[24]. Applying 250 mmHg PO_2 pressure or 70% oxygen inhalation for 5 d or 2.5 atmospheres of hyperbaric oxygen pressure for 150 min/d for 3 consecutive days can lead to resolution of gas collection within a cyst[10,13,24,25]. An urgent laparotomy is necessary in cases of intestinal ischemia, obstruction, intestinal bleeding, or peritonitis[14,16]. Definitive surgery should be performed during a laparotomy if necrosis, perforation, or marked ischemia is observed in the intestine. Furthermore, no additional surgical procedures should be conducted unless other pathology is detected in addition to serosal or subserosal air cysts.

Consequently, clinical suspicion, physician experience, radiological tools, and team spirit are important in terms of the approach to PCI. When and how to treat these patients is the main issue to lower mortality and morbidity.

COMMENTS

Background

Pneumatosis cystoides intestinalis (PCI) is a pathologic condition defined as infiltration of gas into the wall of the gastrointestinal tract.

Research frontiers

The authors retrospectively reviewed the diagnosis and management of seven patients with pneumatosis cystoides intestinalis.

Innovations and breakthroughs

Clinical suspicion, physician experience, radiological tools and team spirit are important in terms of the approach to PCI. When and how to treat these patients is the main issue to lower mortality and morbidity.

Applications

According to authors' opinion, specific treatment is not recommended in asymptomatic patients who are detected to have PCI radiologically and whose examination findings are negative. However, laparotomy is necessary in cases of intestinal ischemia, obstruction, intestinal bleeding or peritonitis

Terminology

The primary and idiopathic or secondary nature of the PCI is determined by considering the medical history and by preoperative examination. Cases with no underlying predisposing disease are considered primary PCI, whereas those accompanying some disease, such as appendicitis, Crohn's disease, pyloric stenosis, necrotizing enterocolitis, peptic ulcers, cystic fibrosis or chronic obstructive lung disease, are regarded as secondary PCI.

Peer review

This is a well written report on a small series of a rare entity. It has some educational value in the presentation and the figures.

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BRIEF ARTICLE

Survival analysis of cholangiocarcinoma: A 10-year experience in Malaysia

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Abstract

AIM: To investigate the clinical features and survival of patients treated for cholangiocarcinoma in our institution and to analyze the factors affecting their survival.

METHODS: This retrospective cohort study assessed patients diagnosed with cholangiocarcinoma between January 1997 and December 2007 at the University Malaya Medical Centre in Malaysia. The clinical data and associated outcomes were collected using a structured proforma.

RESULTS: Of the 69 patients diagnosed with cholangiocarcinoma, 38 (55%) were male; mean patient age was 61 years. Twelve patients (17%) had intrahepatic, 38 (55%) had perihilar and 19 (28%) had distal tumors. Only 12 patients underwent curative surgery, including seven R0 resections. Only one patient died within 30 d after surgery. The overall median survival was 4 mo, whereas the median survival of R0 resected patients was 16 mo. The overall 1-, 2- and 3-year cumulative survival rates were 67%, 17% and 17%, respectively. Survival rates were significantly associated with curative resection (P = 0.002), intrahepatic tumor (P = 0.003), negative margin status (P = 0.013), early tumor stage (P = 0.016), higher tumor differentiation (P = 0.032) and absence of jaundice (P = 0.038). Multivariate analysis showed that tumor location was a significant independent predictor of patient survival.

CONCLUSION: Curative, margin-negative resection of early stage, well-differentiated intrahepatic tumors is associated with improved patient survival.

Key words: Cholangiocarcinoma; Bile duct tumor; Surgery; Malaysia

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INTRODUCTION

Cholangiocarcinoma is a rare malignant neoplasm of biliary tract epithelium, accounting for less than 2% of all human malignancies[1]. Cholangiocarcinoma is the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC)[2] and is associated with

poor patient outcomes. Over the past three decades, however, the worldwide incidence of and mortality from cholangiocarcinoma have steadily increased[3].

Cholangiocarcinoma is difficult to diagnose and is usually fatal, due to its late clinical presentation and the absence of effective non-surgical therapeutic modalities[4]. The incidence of cholangiocarcinoma peaks in patients aged 50-70 years, and there is a slight male predominance[5]. Most patients have unresectable disease at the time of diagnosis and usually die within 6-12 mo from the cancer cachexia, liver failure and biliary sepsis[6]. The 5-year survival rate is low (about 5%), and has remained virtually unchanged over the past 20 years[5,7]. Surgical resection remains the only hope for cure, and radical resection has improved outcomes, although it is also associated with high perioperative morbidity and mortality rates[8]. Since most patients with cholangiocarcinoma present with advanced disease, palliative stenting and chemoradiation are reserved for non-resectable patients, those with recurrence, and those who refuse surgical treatment[3].

Little is known about the survival of cholangiocarcinoma patients in Malaysia. We therefore analyzed the factors affecting survival of patients with cholangiocarcinoma treated at the University Malaya Medical Centre, Malaysia.

MATERIALS AND METHODS

This retrospective cohort study assessed patients diagnosed with cholangiocarcinoma and treated between January 1997 and December 2007 at our center, a tertiary referral center in Malaysia with a specialized hepatobiliary surgery unit and a gastroenterology unit. Patients with a diagnosis of cholangiocarcinoma confirmed histologically by tissue biopsy, and patients without a histologically confirmed diagnosis but with a strong provisional diagnosis by clinical examination, biochemical results and positive endoscopic or imaging [i.e., endoscopic retrograde cholangiopancreatography (ERCP)/magnetic resonance cholangiopancreatography (MRCP) or computed tomography (CT) of the abdomen] results[7] were included. Patients with HCC, tumor at the head of the pancreas and gallbladder carcinoma were excluded.

Combinations of ultrasonography, CT, MRCP, ERCP and percutaneous cholangiography (PTC) were used for tumor diagnosis and staging, and for assessment of resectability. Metastatic disease was evaluated by CT of the thorax, abdomen and pelvis and/or chest radiography (CXR).

Patients were classified into three groups based on the anatomic location of the primary lesion, specifically intrahepatic, perihilar and distal types as proposed by the guidelines for the diagnosis and treatment of cholangiocarcinoma[5]. Intrahepatic tumors were defined as those confined to the liver and not involving the extrahepatic biliary tree. Perihilar tumors were defined as those involving or requiring resection of the hepatic duct bifurcation and were typically located in the extrahepatic biliary tree proximal to the origin of the cystic duct. Distal tumors were defined as extrahepatic lesions located in the peripancreatic region[9].

Patients were staged according to the tumor-node-metastasis (TNM) system[6,10] and assessed for resectability. Variables assessed included therapeutic options (surgical or palliative treatment), operative data, and 30-d postoperative morbidity and mortality. Survival was measured from the date of first presentation to the date of death or last follow-up visit.

Statistical calculations were performed using SPSS version 13.0. Categorical variables were compared using χ^2 tests for association. One-way analysis of variance was used to compare continuous variables among the three groups of patients classified by tumor location. Results were presented as means ± SD, unless otherwise specified. Survival curves were calculated using the Kaplan-Meier method and compared using log-rank tests (Mantel-Cox). Cox proportional hazard models were used to calculate adjusted hazard ratios. A P value < 0.05 was considered significant.

RESULTS

Demography

Of the 69 patients included in this study, 12 (17%) had intrahepatic, 38 (55%) had perihilar and 19 (28%) had distal tumors. Thirty-eight patients were male (55%) and 31 (45%) were female (a male to female ratio, 1.2:1). Mean ± SD patient age was 61 ± 14.2 years (range, 18-91 years), although patients with intrahepatic tumors were younger than those with perihilar and distal tumors (P < 0.05). When subdivided ethnically, 42 patients (61%) were Chinese, 20 (29%) were Malay and 7 (10%) were Indian.

Risk factors

Of the 69 patients, 22 (29%) had a history of chronic cigarette smoking, 13 (19%) each had diabetes mellitus and regular alcohol intake, and 5 (7%) had chronic hepatitis B, with only 1 each (1.4%) having hepatolithiasis and choledochal cyst. None of our patients had other strong risk factors, such as inflammatory bowel disease, primary sclerosing cholangitis or liver fluke infestation.

Clinical evaluation

The most common symptoms among our patients were jaundice (78%), anorexia (57%), weight loss (52%), abdominal pain (44%), abdominal mass (44%), itchiness (25%), vomiting (9%) and fever (7%). Ninety-eight percent of the patients with extrahepatic cholangiocarcinoma were jaundiced (63% of perihilar tumor and 35% of distal tumor patients), whereas only 2% of intrahepatic patients was jaundiced (P < 0.001). No specific symptom was significantly related to any of the three types of cholangiocarcinoma. The median duration of complaints prior to medical consultation was 30 d (range 1-365 d). However, patients with intrahepatic lesions present somewhat later (P

Table 1 Laboratory data on admission and location of tumours

	Total (n = 69)	Intrahepatic (n = 12)	Perihilar (n = 38)	Distal (n = 19)	P value[1]
Total bilirubin (mmol/L)	178 (158)	45 (109)	216 (163)	188 (131)	0.003
Conjugated bilirubin (mmol/L)	140 (122)	31 (87)	170 (123)	151 (102)	0.002
INR	1.2 (0.7)	1.1 (0.1)	1.0 (0.1)	1.6 (1.1)	0.005

[1]Analysis of variance. Values in mean (SD); P value < 0.05 is significant. INR: International normalized ratio.

< 0.05) than those with perihilar or distal lesions.

Investigations

When we assessed laboratory variables in these patients, we found that total bilirubin concentration was significantly higher in patients with perihilar than in those with distal or intrahepatic lesions (P = 0.003) (Table 1). The international normalized ratio (INR) value was also predictive of lesion site, as it was higher in patients with distal than with perihilar or intrahepatic lesions (P = 0.005).

The tumor markers carcinoembryonic antigen, alpha-fetoprotein and carbohydrate antigen 19-9 were measured in about 50% of these patients, but none of them significantly correlated with tumor location.

Imaging

Ultrasound was the first line non-invasive imaging method used in 45 (65%) patients, detecting 50%-76% of obstructed biliary systems. Almost 91% of these patients underwent CT scans to further assess the extent of pathology, including liver masses, lymph nodes and the involvement of major vessels. We found that about 20% of the abdominal lesions were not detected by ultrasound alone, but were detected on CT scan. Only four patients underwent MRCP.

In 58 (84%) patients, ERCP was the first invasive method used to assess the obstructed biliary system. ERCP showed that 51 (74%) patients had strictures at various levels of their biliary trees. Plastic stents were inserted successfully into 44 (76%) of these patients to relieve their biliary obstructions, whereas the other seven (14%) failed in stent insertion; five later underwent PTC drainage. Cytology brushing of suspicious strictures was performed in 22 (38%) patients, with 10 (45%) being positive for cancer.

Preoperative staging for resectability was predicted based on ultrasound, CT scan, MRCP and CXR. None of these patients underwent staging laparoscopy because it was not a routine practice in our center.Based on the American Joint Committee on Cancer TNM staging system, 23 (33%) patients had stage 1 tumors, 7 (10%) had stage 2, 17 (25%) had stage 3 and 22 (32%) had stage 4. Stages 3 and 4 were considered unresectable.

Surgical treatment

Although preoperative staging indicated that 30 (43%) patients were candidates for surgical resection, only 22 (32%) patients underwent laparotomy with curative intent. Three patients (and their relatives) refused surgery, citing age as their primary concern (mean age 78 years), whereas five were excluded from surgery due to age (mean 71 years) and/or associated comorbidity. At laparotomy, 10 patients had extensive local disease and/or hepatic metastasis that precluded resection. The remaining 12 patients underwent potentially curative resection (resectability rate, 55%). All except one with evidence of biliary obstruction underwent preoperative biliary drainage either by endoscopic stenting or percutaneous transhepatic biliary drainage (PTBD). The type of surgery depended on the location of the tumor (Table 2). In general, intrahepatic tumors were treated by hepatic resection; perihilar lesions by excision of the extrahepatic biliary tree and lymph node dissection, with or without hepatic resection; and distal tumors by Whipple pancreaticoduodenectomy. Biliary reconstructions were mostly by Roux-ex-Y hepaticojejunostomy. All operations were performed by trained hepatobiliary surgeons.

Table 2 Types of surgical resection

Types of surgery	Patients (n = 12)
Left hemihepatectomy	2
Right hemihepatectomy	1
Segmental hepatic resections	2
Extrahepatic bile duct resection	5
Whipple's procedure	2

Surgical morbidity and mortality

Complications occurred in patients with all three tumor types, but the differences were not statistically significant. Three patients developed post-operative ileus, which resolved after conservative treatment. Two patients had intra-abdominal hemorrhage. One had undergone a Whipple procedure and was re-explored within 24 h because of portovenous bleeding; unfortunately, this patient died the next day. The second patient did not require intervention and was treated with blood transfusion and correction of coagulopathy. Three patients developed intra-abdominal collection, later complicated by abscesses, including one caused by bile leakage from the anastomosis. None of these patients, however, required surgical or percutaneous intervention. Two patients had surgical site infections, including one with anastomotic stenosis and the other with deep venous thrombosis (DVT) despite DVT prophylaxis. Only one patient died of complications within 30 postoperative days; hence the perioperative mortality rate was 8%.

Of the 10 patients who underwent surgery but were found to have advanced inoperable disease at laparotomy, 3 underwent a palliative bypass procedure, consisting of hepaticojejunostomy and gastrojejunostomy, whereas four underwent gastrojejunostomy alone. Two patients underwent cholecystectomy and biliary stent insertion, whereas one underwent only laparotomy and biopsy.

Table 3 Tumour histology, degree of differentiation, diameter, margin, perineural and lymph node involvements by tumour location *n* (%)

	Total (*n* = 12)	Intrahepatic (*n* = 3)	Perihilar (*n* = 6)	Distal (*n* = 3)	*P* value[1]
Tumour histology					
Adenocarcinoma	11 (92)	2 (18%)	6 (55%)	3 (27)	
Other	1 (8)	1 (100%)	0	0	
Degree of differentiation					
Well	5 (42)	2 (40%)	2 (40%)	1 (20)	
Moderate	6 (50)	1 (17%)	3 (50%)	2 (34)	
Poor	1 (8)	0	1 (100%)	0	
Tumour diameter, cm					
Size, mean ± SD	9.6 ± 15.2	27.1 ± 24.3	2.8 ± 2.2	5.7 ± 15.2	0.048[1]
Margin					
Negative	7 (58)	3 (43)	3 (43)	1 (14)	
Positive	5 (42)	0	3 (60)	2 (40)	
Lymph node involvement					
Negative	8 (67)	2 (25)	4 (50)	2 (25)	
Positive	4 (33)	1 (25)	2 (50)	1 (25)	
Perineural involvement					
Negative	5 (42)	3 (60)	2 (40)	0	
Positive	7 (58)	0	4 (57)	3 (43)	
Lymphovascular invasion					
Negative	7 (58)	2 (29)	4 (57)	1 (14)	
Positive	5 (42)	1 (20)	2 (40)	2 (40)	

[1]Analysis of variance. *P* value < 0.05 is significant.

Tumor characteristics

Of the 12 patients who underwent curative resection, 11 (92%) had adenocarcinoma and one had a papillary adenocarcinoma. The mean ± SD tumor diameter was 9.6 ± 15.2 cm (range, 0.5 cm-55 cm). Intrahepatic tumors were larger than perihilar and distal tumors ($P < 0.05$). Five (42%) patients had well-differentiated adenocarcinoma and the others had moderately or poorly differentiated adenocarcinoma. Seven (58%) patients had perineural involvement, 5 (42%) had lymphovascular invasion and 4 (33%) had regional lymph node metastases. Seven (58%) patients were resected with negative margins (R0 resection), whereas the other 5 (42%) had microscopically positive margins. Table 3 summarizes the characteristics of these tumors.

Palliation

Forty-seven (68%) patients did not undergo surgery but received palliative treatment, including 31 (66%) who underwent palliative biliary drainage by endoscopic stenting, 23 (49%) who underwent PTBD with or without concurrent stenting and 13 who underwent both. Sixteen patients had their stents changed on subsequent follow-up, of whom, nine had self-expanding metal stents. Three (6%) patients received palliative chemotherapy alone, using a variety of chemotherapeutic agents (5-fluorouracil, cisplatin, gemcitabine). Three (6%) patients were too ill and hence received best supportive care.

DISCUSSION

We have described our experience managing patients with cholangiocarcinoma, a rare type of tumor. The incidence of this tumor is likely increasing in Malaysia. The latest National Cancer Registry 2003-2005 Peninsular Malaysia has classified cholangiocarcinoma into the category of liver and gallbladder cancers rather than as a separate tumor. The incidence of liver cancer in Malaysia has been reported to be 3.6% for males and 1.2% for females, while the rates of gallbladder cancer were 0.8% and 0.7%, respectively. Morphologically, about 2.2% of these liver cancers and 33.9% of these gallbladder cancers were cholangiocarcinomas. The incidence of both liver and gallbladder cancers was higher for Chinese than for Malays and Indians.

Cholangiocarcinoma is best classified according to its anatomical location into intrahepatic, perihilar and distal tumors[9,11,12]. Most (40%-60%) tumors are perihilar or Klatskin tumors, with 20%-30% being distal and 10% being intrahepatic tumors[9,13]. Our findings were similar to these rates, in that 17% of tumors were intrahepatic, 54% were perihilar and 29% were distal.

The demographic characteristics of our patients were comparable to those of patients in other larger series. Mean patient age was 61 years (range, 18-91 years), while a review of 294 patients with cholangiocarcinoma by Nakeeb *et al*[9] showed a mean age of 62.2 years and a review by DeOliveira *et al*[13] of 564 patients showed a median age of 65 years. We found, however, that patients with intrahepatic tumors were significantly younger than those with perihilar or distal tumors. We observed a slight male predominance, with a male to female ratio of 1.2:1, similar to the ratios of 1.2:1[9] and 1.38:1 reported previously[13].

Of these patients, 32% presented in 1997-2002, whereas 68% presented in 2003-2007. We observed similar increases in the number of patients with primary tumors, suggesting that these higher rates may be due to improvement in tumor detection, ERCP proficiency, or increased awareness of the availability of local surgical expertise.

Only a few patients in our series had strong risk factors such as chronic hepatitis B, hepatolithiasis and choledochal cyst[14-16]. None had a history of primary sclerosing cholangitis[14] or liver fluke infestation[17], the latter of which is not endemic in Malaysia. Chronic cigarette smoking, regular alcohol intake and diabetes mellitus were among the risk factors[18,19] observed in our population, but none of these risk factors was associated with any particular tumor site.

The most common symptom observed in these patients was jaundice. Jaundice was significantly more common in patients with extrahepatic (i.e., perihilar and distal) than with intrahepatic cholangiocarcinoma ($P < 0.001$). This was confirmed by laboratory results showing that the concentration of bilirubin was significantly higher in patients with perihilar (extrahepatic) than in those with intrahepatic tumors ($P = 0.002$). Similar results have been reported previously[13,20]. We also found that INR was higher in patients with distal tumors ($P =$

Table 4 Univariate analysis of different variables in relation to survival

Variables	Survival rates (%)			*P* value
	1-year	2-year	3-year	
Jaundice				0.038
Absent	40	19	9	
Present	19	6	2	
Staging				0.016
Stage 1	43	17	9	
Stage 2	43	14	0	
Stage 3	12	4	0	
Stage 4	5	0	0	
Surgery (curative or palliative)				0.017
No	13	10	0	
Yes	45	9	9	
Curative resection				0.002
No	14	8	0	
Yes	67	17	17	
Type (among resected patient)				0.003
Intrahepatic	100	33	33	
Perihilar	67	17	17	
Distal	33	0	0	
Type (among all patient)				0.027
Intrahepatic	50	23	11	
Perihilar	18	6	3	
Distal	16	5	0	
Histology				0.032
Well	80	40	40	
Moderate and poor	57	0	0	
Margin involvement				0.013
Negative	86	29	29	
Positive	40	0	0	

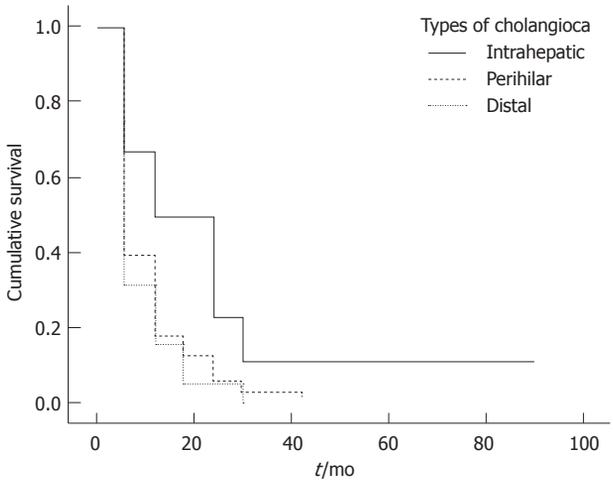

Figure 1 Overall survival by types of cholangiocarcinoma regardless of surgery or not, *P* = 0.027.

0.005). Obstructive jaundice may decrease the concentrations of vitamin K-dependent coagulation factors, resulting in aberrant coagulation profiles[5]. However, none of the other symptoms or blood parameters we assayed was significantly related to tumor location.

Many of our patients initially underwent ERCP, not MRCP. ERCP enables cytological brushing and can decompress the obstructed biliary system[6]. Although we found that the success rate of internal stent placement for drainage was high, PTBD may also play a role, especially when the endoscopic approach has failed. PTBD can also be used to visualize proximal biliary tumors and anatomy[5]. Failure of biliary decompression may result in an infected biliary system and further risks of liver failure and sepsis[21]. These risks may be prevented by decompression of an obstructed biliary system in patients with potentially resectable cholangiocarcinoma, although a series by Figueras *et al*[22] demonstrated no significant differences in morbidity and mortality between patients with and without preoperative biliary drainage. None of our patients who underwent hepatic resection developed postoperative liver failure.

Despite improved diagnostic methods and a relatively early presentation (median duration of symptoms, 30 d), only about one-third of our patients (32%) underwent surgery, and only 12 underwent curative resection. More than half of our patients (58%) were considered to have advanced unresectable disease, whereas the remaining patients were considered unsuitable for surgery because of comorbidity and/or advanced age, findings similar to those observed previously[7]. Our overall resectability, 55% (12/22), was similar to previously reported rates 18%-70%[23]. Postoperative complications were not associated with tumor location. In high volume centers with considerable experience, the operative mortality and morbidity rates varied from 6%-14% and 32%-65%, respectively[24-26]. Our 30-d postoperative mortality and morbidity rates were similar, 8% and 67%, respectively.

Patients who underwent surgical resection had a definite survival advantage over those who did not, confirming that surgical resection is the best treatment available for patients with cholangiocarcinoma and providing further evidence that potentially resectable patients should be referred early to a specialized surgical team[7,10]. The median survival time for the 12 patients who underwent curative resection was 16 mo, compared with 3 mo for the 57 patients who did not undergo curative resection (P = 0.002). The 1-, 2- and 3-year cumulative survival rates in patients who underwent resection were 67%, 17% and 17%, respectively, significantly higher than the 14%, 8% and 0%, respectively, in those who did not. Furthermore, complete surgical resection with histologically negative margins offers the best chance for cure and long-term survival[27-30].

Univariate log-rank analyses of tumor related variables in Table 4 showed that absence of jaundice (P = 0.038), tumor location (P = 0.027) (Figure 1)[17], curative resection (P = 0.002) (Figure 2)[31], early tumor stage (P = 0.016)[32], negative margin status (P = 0.013) (Figure 3)[17,32,33] and higher degree of tumor differentiation (P = 0.032) (Figure 4)[17] were significant predictors of longer survival. In contrast to previous reports, regional lymph node metastasis[28] and perineural invasion[34] were not associated with survival in resected patients. Moreover, we found that age, gender, race and risk factors were not predictors of survival.

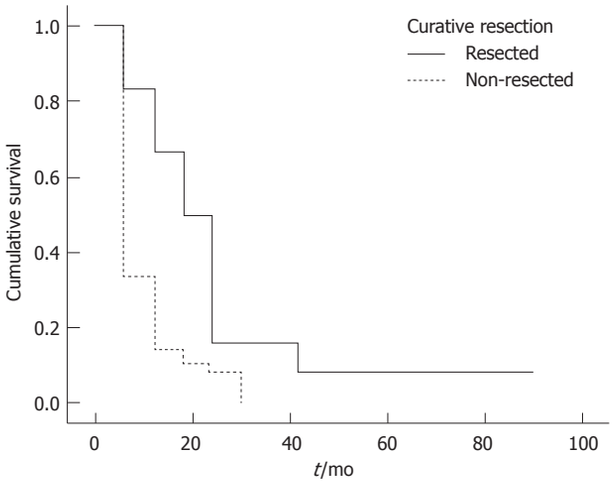

Figure 2 Cumulative survival curves after curative resection, *P* = 0.002.

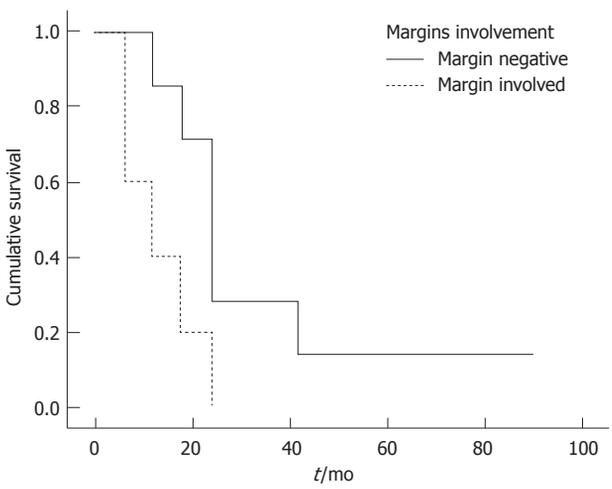

Figure 3 Cumulative survival curves after resection according to histologic margin clearance, *P* = 0.013.

Multivariate analysis showed that tumor location was the only independent predictor of long-term survival. Survival was significantly lower in patients with perihilar than in those with intrahepatic and distal tumors (HR = 0.016, 95% CI: 0.01-0.607; $P = 0.026$), in agreement with the findings of other series showing that patients with intrahepatic tumors had the best survival[17,2]. This could be explained by the fact that, compared with extrahepatic tumors, intrahepatic tumors are characterized by different epidemiology and tumor biology, younger age ($P < 0.05$), and lower rate of tumor negative margins[10,17].

For the majority of patients with cholangiocarcinoma who cannot undergo curative resection, palliative treatment to relieve jaundice, pruritus and cholangitis and to avoid liver failure becomes the priority. This can be achieved surgically *via* biliary-enteric bypass or stent placement *via* PTBD or ERCP[33]. We found however, that neither palliative surgical bypass nor biliary drainage had significant survival benefits, in agreement with previous findings by Prat *et al*[34] Both groups had a median survival of 3 mo.

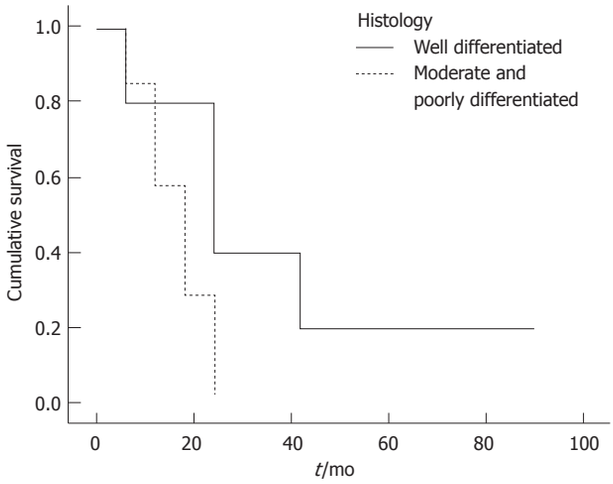

Figure 4 Overall survival according to degree of tumour differentiation, *P* = 0.032.

A novel palliative therapy for unresectable cholangiocarcinoma, photodynamic therapy (PDT), has shown promising benefits in terms of patient survival, cholestasis and quality of life[35]. PDT utilizes the intravenous photosensitizer sodium porfimer, which accumulates in tumor tissues. Upon illumination of the tumor bed by a specific endoscopic light, the porfimer becomes activated and forms oxygen free radicals, resulting in tumor necrosis[36].

The major limitation of our study was its involvement of patients at a single center. Therefore, our findings may not be representative of patients with cholangiocarcinoma throughout Malaysia.

The incidence of cholangiocarcinoma is increasing throughout Malaysia, as shown by the increase in the number of patients diagnosed per year throughout our study period. This may be due to an increased detection rate and to increases in referrals to our center. Curative surgical resection with clear histological margins of early stage well-differentiated intrahepatic tumors is associated with improved long-term survival. Further prospective randomized studies involving multiple centers are warranted.

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COMMENTS

Background

Cholangiocarcinoma is a rare malignant cancer of the bile duct with poor prognosis. This cancer is difficult to diagnose and often the patient presented late when curative surgical resection that can provide the chance for cure, is not feasible. Any person in his or her fifth decade with jaundice and significant

weight loss should raise a suspicion of this cancer. Despite its rarity, the incidence of this cancer has been steadily increasing worldwide.

Research frontiers

Many studies have concurred that early detection together with radical surgery will increase the survival period of these patients. A research article to be published in the *World Journal of Gastroenterology* has further emphasized the importance of early diagnosis and early referral to a specialized surgical centre with vast experience in the management of this cancer.

Innovations and breakthroughs

The authors have analysed various factors that affect the survival of their cholangiocarcinoma patients treated over a 10-year period. It was found that the survival period will improve if complete tumour excision is performed whereby the margins are tumour-free on histology. The outcome is also favourable if surgery is performed early when the abnormal cells are still at their early grade and have not spread elsewhere. Despite a range of palliative procedures available for inoperable cancer, none could surpass the result of a successful surgical treatment. These have further consolidated similar findings and recommendations of early aggressive surgery from other larger centres in the West and Far East.

Applications

The study also shows that cholangiocarcinoma is an important health problem in Malaysia. Although the increase in number of patient being treated may not truly reflect a true increase in incidence, this would mean more need to be done to address such problem. This study provides a framework for future studies in Malaysia and hopefully stimulates other groundbreaking research in this region especially concerning the epidemiology and pathophysiology of this fatal cancer.

Peer review

This 10-year retrospective review defines treatment modalities and survival statistics for cholangiocarcinoma in a Malaysian Referral Hospital. Not surprisingly, patients who underwent R-O resections had higher cumulative survival rates than patients palliated surgically, endoscopically or by percutaneous transhepatic biliary drainage. This manuscript is worthy of publication.

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BRIEF ARTICLE

Thalidomide-based multidisciplinary treatment for patients with advanced hepatocellular carcinoma: A retrospective analysis

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Abstract

AIM: To evaluate the efficacy of thalidomide in combination with other therapies to treat patients with advanced hepatocellular carcinoma (HCC).

METHODS: We performed a retrospective analysis of all patients with HCC who were treated with thalidomide for at least two months. The medical records of patients with HCC who were treated at our institution between April 2003 and March 2008 were reviewed. Image studies performed before and after treatment, tumor response, overall survival, and the decrease in α-fetoprotein (AFP) levels were evaluated.

RESULTS: A total of 53 patients with HCC received either 100 or 200 mg/d of thalidomide. The patient population consisted of 9 women and 44 men with a median age of 61 years. Thirty patients (56.6%) were classified as Child-Pugh A, and 12 patients (22.6%) were classified as Child-Pugh B. Twenty-six patients had portal vein thrombosis (49.1%), and 25 patients had extrahepatic metastasis (47.1%). The median duration of thalidomide treatment was 6.0 mo. Six of the 53 patients achieved a confirmed response (11.3%), one achieved a complete response (1.9%) and 5 achieved a partial response (9.4%). The disease control rate (CR + PR + SD) was 28.3% (95% CI: 17.8-42.4), and the median overall survival rate was 10.5 mo. The 1- and 2-year survival rates were 45% and 20%, respectively. Only one complete response patient showed an improved overall survival rate of 66.8 mo. Sixteen patients (30.2%) showed more than a 50% decrease in their serum AFP levels from baseline, indicating a better response rate (31.3%), disease control rate (43.8%), and overall survival time (20.7 mo). The therapy was well tolerated, and no significant toxicities were observed.

CONCLUSION: Thalidomide was found to be safe for advanced HCC patients, demonstrating anti-tumor activity including response, survival, and AFP decreases of greater than 50% from baseline.

Key words: Thalidomide; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, the fifth most common malignancy worldwide (with over 700 000 new cases per year), and the third most common cause of cancer deaths[1]. In

Taiwan, HCC, which ranks second among the major types of cancer in the list of cancer-related mortalities, is responsible for approximately 7000 to 8000 deaths per year[2]. Unfortunately, most patients seek treatment when the disease is beyond curative treatment (surgery or percutaneous ablation), and palliative care is the only alternative. According to the Barcelona Clinic Liver Cancer (BCLC) staging classification[1] and treatment schedule, chemoembolization is the best option for intermediate-stage patients. However, for advanced-stage patients, no standard treatment was established until 2007. Systemic chemotherapy is generally ineffective and is associated with significant toxicity because hepatic function is often impaired by underlying cirrhosis that is often accompanied by hypersplenism and peripheral cytopenia[3]. Fortunately, after the positive results of the Study of Heart and Renal Protection (SHARP) trials[4], a new treatment, sorafenib, was approved for advanced-stage patients, which offers major improvements in overall survival and time to progression compared to placebo. There are many new modalities of treatment with more favorable therapeutic indices that are suitable for patients with advanced HCC. HCC is a hypervascular tumor that is one of the most antiangiogenic and angiogenesis-dependent tumors[5,6]. Consequently, it is reasonable to hypothesize that antiangiogenesis therapy may inhibit the growth of HCC. A number of antiangiogenic agents have been developed, including thalidomide, which is a glutamic acid derivative that was first described in 1953 when it was labeled as a sedative and anti-emetic agent. However, it was withdrawn from the European market 30 years ago because of its teratogenic effects[7]. Recently, oral thalidomide has been shown to inhibit basic fibroblast growth factor- and vascular endothelial growth factor-induced angiogenesis of cancer cells[8,9]. Studies published on the efficacy of thalidomide in advanced hepatocellular carcinoma have reported modest responses to therapy with acceptable toxicity[10-12]. Treatment of patients with HCC continues to present a major challenge. We retrospectively analyzed our records of HCC patients who received thalidomide in combination with other therapies to determine whether thalidomide was effective.

MATERIALS AND METHODS

Patients

Between April 2003 and March 2008, 53 patients with HCC were treated for at least two months with either 100 or 200 mg/d of thalidomide (50 mg/capsule, TTY Biopharm Co. Ltd., Taipei, Taiwan) at Changhua Christian Medical Center in Taiwan. HCC was diagnosed by histological examination and imaging findings. The diagnosis of HCC was confirmed by histological examination or the presence of all of the following criteria: (1) pathological diagnosis of HCC; (2) cirrhotic liver with a tumor size greater than 2 cm plus one dynamic image [computed tomography (CT) or magnetic resnane iamge (MRI)] or alpha fetoprotein (AFP) > 200 ng/mL; (3) cirrhotic liver with a tumor size of 1-2 cm plus two dynamic images (CT + MRI); and (4) non-cirrhotic liver greater than 2 cm plus one dynamic image (CT or MRI) and AFP > 200 ng/mL. The inclusion criteria were as follows: (1) advanced HCC (surgically unresectable); (2) failed previous local therapy, such as radiotherapy, hepatic arterial chemoembolization, radiofrequency ablation, or percutaneous interventional therapy; and (3) distant metastasis (lung, lymph node, or bone) that is not eligible for curative surgery and radiotherapy or locoregional therapy failure [e.g., transarterial chemoembolization (TACE), recurrence-free interval or percutaneous ethanol injection (PEI)]. All patients had bidimensionally measurable disease that was staged by the pathological tumor-node-metastasis (TNM) system, the Okuda system, and the BCLC parameters for HCC. The demographic data, details of the primary tumors, serum AFP levels, dates of recurrence, length of survival, and last follow-up dates were analyzed retrospectively. The responses to thalidomide were determined by CT performed according to the Response Evaluation Criteria in Solid Tumor Guidelines[13], and the AFP levels were also analyzed before and after thalidomide treatment. Overall survival was calculated from the date of the start of chemotherapy and analyzed by the Kaplan-Meier method. Follow-up data were obtained for all patients until the time of their death or the last follow-up.

RESULTS

Patient and demographic characteristics

A total of 53 patients with HCC were available for analysis, and their demographic characteristics are shown in Table 1. The patient population included 9 females (17.0%) and 44 males (83.0%) with a median age of 61 years (range, 29-88 years). Of the 53 patients, 10 had not received prior treatment or therapy. Pretreatment curative surgery had been performed on 12 patients (22.6%), transarterial embolization on 13 patients (24.5%), TACE on 16 patients (30.2%), radio frequency ablation on 10 patients (18.9%), and radiotherapy (RT) on 10 patients (18.9%). Twenty-six patients had portal vein thrombosis (49.1%), and 25 patients had extrahepatic metastasis (47.2%). The prevalence of hepatitis B was 56.5% (30/53), that of hepatitis C was 37.7% (20/53), and that of concomitant hepatitis was 1.9% (1/53). Of the 53 patients, most patients had TNM stage Ⅳ (45.3%), Okuda stage I (51.9%), and BCLC stage C (71.2%). There were 22 patients (41.5%) whose serum AFP levels were greater than 400 ng/mL above the baseline. The liver functions of the majority of patients were classified as Child-Pugh A (56.6%), and the median duration of treatment was 6.0 mo (range, 1.5-53.9 mo) (Table 1).

Efficacy

Of the 53 patients, one had a complete response (CR, 2.9%) to thalidomide, five had a partial response (PR, 9.4%) and nine were classified as stable disease (SD,

Table 1 The clinical characteristics of 53 patients with hepatocellular carcinoma

Characteristic	*n* (%)
Age, yr (median)	61 (range, 29-88)
Sex	
Male	44 (83.0)
Female	9 (17.0)
Type of hepatitis	
Hepatitis B	30 (56.6)
Hepatitis C	20 (37.7)
Hepatitis B + C	1 (1.9)
Child-Pugh classification	
A	30 (56.6)
B	12 (22.6)
C	1 (1.8)
TNM stage	
Ⅰ	0 (0)
Ⅱ	6 (11.2)
ⅢA	11 (20.8)
ⅢB	3 (5.7)
ⅢC	6 (11.2)
Ⅳ	24 (45.3)
Okuda stage	
Ⅰ	27 (51.9)
Ⅱ	16 (30.8)
Ⅲ	1 (1.9)
BCLC stage	
A	2 (3.9)
B	7 (13.5)
C	37 (71.2)
D	1 (1.9)
Extrahepatic metastasis	
Yes	25 (47.2)
No	28 (52.8)
Portal vein thrombosis	
Yes	26 (49.1)
No	26 (49.1)
Unknown	1 (1.8%)
Site of extrahepatic metastasis	
Lung	11 (21.2)
Bone	6 (11.5)
Brain	1 (1.9)
Others	7 (13.5)
Prior therapy	
Surgery	12 (22.6)
TACE	16 (30.2)
TAE	13 (24.5)
Radiation therapy	10 (18.9)
RFA	10 (18.9)
No therapy	10 (18.9)
Duration of treatment, mo	
Median	6.0 (range, 1.5-53.9)
AFP level	
> 400 ng/mL	31 (58.5)
< 400 ng/mL	22 (41.5)

BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization; TAE: Transarterial embolization; RFA: Radiofrequency ablation; AFP: Alpha fetoprotein; TNM: Tumor node metastasis.

17.0%). The remaining 38 patients had disease that continued to progress after the thalidomide treatments. The objective response rate was 11.3% (95% CI: 4.3-23.0), and the disease control rate (CR + PR + SD) was 28.3% (95% CI: 17.8-42.4). The median overall survival rate was 10.5 mo (95% CI: 6.9-23.3). The 1- and 2-year survival rates were 45% and 20%, respectively. Sixteen patients (30.2%) showed more than a 50% decrease in their serum AFP levels below the baseline and showed a better response rate (31.3%), disease control rate (43.8%), and overall survival time (20.7 mo) (Table 2, Figure 1). The prognostic factors for the response rate, disease control rate, and overall survival in HCC patients receiving thalidomide are listed in Table 3. Multivariate analysis showed that almost all of these patients qualified as having independent prognostic factors for the efficacy analysis. The only significant difference in the efficacy activity was an AFP decrease of > 50% after treatment. The median overall survival time of the patients who registered > 50% AFP decrease was 20.7 mo, with a response rate of 31.3% and a disease control rate of 43.8%. The median overall survival time of those patients with a < 50% AFP decrease was 7.1 mo, with a response rate of 2.7% and a disease control rate of 21.6% (Table 3, Figure 1). Table 4 is a comparison of the patients who responded and the patients whose disease progressed. Patients in the CR + PR + SD group had a significantly longer survival time (33.3 mo) than those in the progressive disease (PD) group (6.9 mo, $P < 0.003$) (Figure 1).

Table 2 Efficacy results of thalidomide

Overall objective response, *n* = 53, (%)	
CR	1 (2.9)
PR	5 (9.4)
SD	9 (17.0)
PD	38 (44.1)
Objective response rate	6 (11.3), 95% CI: 4.3-23.0
Disease control rate	15 (28.3), 95% CI: 17.8-42.4
Overall survival, mo	
Median	10.5, 95% CI: 6.9-23.3
1-year survival	(45)
2-year survival	(20)
A decrease in AFP > 50% after treatment	
Yes	16 (30.2)
No	37 (69.8)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; AFP: Alpha fetoprotein.

DISCUSSION

Thalidomide has been used in the treatment of advanced HCC patients. Hsu *et al*[10] reported an overall response rate of 6.3% with an overall survival time of 18.7 wk when an escalating dose (100-600 mg/d) of thalidomide was used for the treatment of advanced HCC. Patt *et al*[14] also showed a 5% overall response rate with a 6.8-mo overall survival time when a high dose (400-1000 mg/d) of thalidomide was used. In a phase Ⅱ study[12], high-dose (200-800 mg/d) single-agent thalidomide demonstrated a response rate of 3.9% with an overall survival time of 123 d. The first retrospective study to analyze the efficacy and tolerability of fixed low-dose thalidomide in the treatment of advanced HCC patients[15]

Table 3 Prognostic factors for efficacy analysis in hepatocellular carcinoma patients receiving thalidomide

Variables		*P* value
Overall response rate *n* (%)		
Child-Pugh classification		
A	3/30 (10.0)	1.000[1]
B and C	3/23 (13.0)	
Okuda staging		
Stage 1	2/27 (7.4)	0.344[1]
Stage 2	3/16 (18.8)	
AFP level		
> 400 ng/mL	1/31 (3.2)	0.071[1]
< 400 ng/mL	5/22 (22.7)	
A decrease in AFP > 50% after treatment		
Yes	5/16 (31.3)	0.007[1]
No	1/37 (2.7)	
Disease control rate *n* (%)		
Child-Pugh Classification		
A	6/30 (20.0)	0.218[1]
B and C	9/23 (39.1)	
Okuda staging		
Stage 1	6/27 (22.2)	0.719[1]
Stage 2	5/16 (31.3)	
AFP level		
> 400 ng/mL	7/31 (22.6)	0.357[1]
< 400 ng/mL	8/22 (36.4)	
A decrease in AFP > 50% after treatment		
Yes	7/16 (43.8)	0.071[1]
No	8/37 (21.6)	
Overall survival, mo		
Child-Pugh Classification		
A	8.8	0.922[2]
B and C	10.8	
Okuda staging		
Stage 1	22.2	0.075[2]
Stage 2	6.9	
AFP level		
> 400 ng/mL	10.8	0.679[2]
< 400 ng/mL	6.5	
A decrease in AFP > 50% after treatment		
Yes	20.7 (95% CI: 1.7-NA)	0.307[2]
No	7.1 (95% CI: 6.3-24.3)	

[1]*P* value was calculated by Fisher's exact test; [2]*P* value was calculated by Log-rank test. AFP: Alpha fetoprotein; NA: Not assessable.

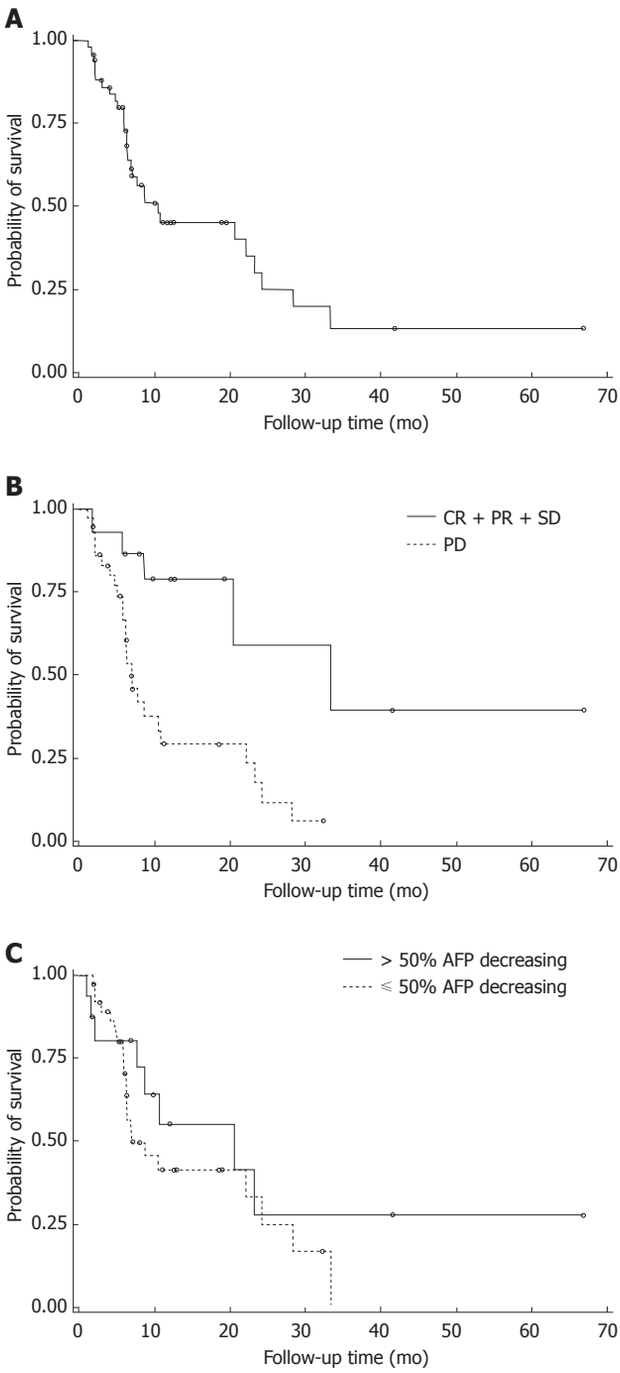

Figure 1 Kaplan-Meier analysis of the survival time in all advanced hepatocellular carcinoma patients (A), in the subgroup of disease stabilization (B), and in the subgroup of > 50% decrease in alpha fetoprotein (C). CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; AFP: Alpha fetoprotein.

showed that low-dose thalidomide has a comparable single-agent activity (response rate of 5%, with an overall survival time of 4.3 mo) but fewer treatment-related toxicities than high-dose thalidomide when treating advanced HCC patients. Patients treated with low-dose thalidomide have similar overall survival times compared to patients treated with chemotherapeutic agents, with a far better toxicity profile and less hematological toxicity (no grade 3/4 neutropenia or thrombocytopenia)[15,16]. The largest randomized phase Ⅲ trial for HCC (the SHARP trial) showed better progression free survival and overall survival times with sorafenib than with placebo[4]. The primary drug-related adverse events were dermatological (constitutional and hand-foot skin reactions) and gastrointestinal[4,17]. The toxicity of sorafenib is a serious problem because approximately 50% of the patients had to interrupt or stop their treatment because of sorafenib-induced toxicity. The tolerance of low-dose thalidomide in HCC patients may be worth further investigation.

The treatment of hepatoma with thalidomide appears to be feasible. A complete response was rare with thalidomide treatment of HCC; the PR rate was 5%-10%, and the SD rate was approximately 37%[10,12,14], depending on the duration of observation, cancer stage, and the definition of stability. In our study, one patient had complete remission; the PR rate was 9.4%, and the SD rate was 17%. One CR patient received thalidomide alone after a TACE therapy failure; the duration of the treatment

Table 4 Comparison of patients who responded and patients with progressive disease

Characteristic	CR	PR	SD	PD	CR + PR + SD	*P* value[a]
AFP level						0.357
> 400 ng/mL	0 (0)	1 (3.2)	6 (19.4)	24 (77.4)	7 (22.6)	
< 400 ng/mL	1 (4.6)	4 (18.2)	3 (13.6)	14 (63.6)	8 (36.4)	
A decrease in AFP > 50% after treatment	1 (6.3)	4 (25.0)	2 (12.5)	9 (56.3)	7 (43.8)	0.182
Overall survival, mo	66.8	NA	20.7 (95% CI: 20.7-33.3)	6.9 (95% CI: 6.3-10.8)	33.3 (95% CI: 20.7-NA)	0.003[b]

AFP: Alpha fetoprotein; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NA: Not assessable. [a]$P < 0.05$ between the two groups (CR + PR + SD *vs* PD); [b]$P = 0.0003$ (less than 0.005) between progress disease and CR + PR + SD.

was 53.9 mo, the patient had no recurrence, and he is still alive (66.8 mo post-treatment). The most interesting finding was the AFP decrease from 11 005.3 ng/mL at diagnosis to < 20 ng/mL (Table 4). Of the 5 patients with partial responses, 2 had prior TACE treatments, 2 had RT, 1 had PEI and 1 had systemic therapy. The median survival time among these patients was 502 d (range, 248-1263 d), and 3 of them are still alive. The median survival time of patients with stable disease was 412 d (range, 60-1013 d).

Patients in the CR + PR + SD group had a significantly longer survival time (33.3 mo) than those in the PD group (6.9 mo, $P < 0.003$). Thalidomide may offer HCC stabilization and prolong survival, especially in patients with stabilization. Survival time should be the focus of future clinical trials of thalidomide therapy. In this study, we evaluated the clinical implication of the AFP tumor marker response in assessing the therapeutic effects of thalidomide in HCC. The results showed that the AFP response was an independent prognostic factor for the response rate, disease control rate, and overall survival time. We also identified patients with more than or less than a 50% decrease in serum AFP levels from the baseline, which made a significant difference in their response rates (31.3% *vs* 2.7%, $P = 0.007$). There was a better trend in the disease control rate (43.8%) and overall survival time (20.7 mo) when there was greater than a 50% AFP decrease (Table 3). The AFP response may correlate with the biological response and, consequently, predict the survival benefits of thalidomide in HCC.

In conclusion, thalidomide has shown modest clinical activity, including response and survival, and was safely administered to patients with advanced HCC. Because the present study is retrospective in nature with a relatively small number of patients, a larger, randomized phase Ⅱ/Ⅲ study is needed to clearly define the role of single-agent thalidomide in the treatment of HCC as an alternative to the expensive molecular-targeted therapies.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, the fifth most common malignancy worldwide (with over 700 000 new cases per year), and the third most common cause of cancer deaths. However, for advanced-stage patients, no standard treatment was established until the positive result of the Study of Heart and Renal Protection study. However, there are many new modalities of treatment with more favorable therapeutic indices that are suitable for patients with advanced HCC.

Research frontiers

HCC is a hypervascular tumor that is one of the most antiangiogenic and angiogenesis-dependent tumors. Recently, thalidomide was shown to inhibit the angiogenesis of cancer cells and studies have reported modest responses to this therapy in advanced HCC. The authors retrospectively analyzed the records of HCC patients who received thalidomide in combination with other therapies to determine whether thalidomide was effective.

Innovations and breakthroughs

Studies published on the efficacy of thalidomide in advanced hepatocellular carcinoma have reported modest responses to the therapy with acceptable toxicity. Some of them highlighted the alpha fetoprotein (AFP) tumor marker response in assessing the therapeutic effects of thalidomide in HCC. In this study, the authors concluded thalidomide showed modest clinical activity, including response and survival, and was safely administered to patients with advanced HCC. Furthermore, they also identified patients with more than or less than a 50% decrease in serum AFP levels from the baseline, which made a significant difference in their response rates.

Applications

The results showed that the AFP response was an independent prognostic factor for the response rate, disease control rate, and overall survival time. The AFP response may correlate with the biological response and, consequently, predict the survival benefits of thalidomide in HCC.

Peer review

This is an interesting and well written manuscript summarising the effects of thalidomide on HCC patients in a retrospective study.

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BRIEF ARTICLE

A pharmacodynamic model of portal hypertension in isolated perfused rat liver

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Abstract

AIM: To develop a pharmacodynamic model of portal hypertension from chronic hepatitis.

METHODS: Pathological changes and collagen depositions were analyzed using morphometry to confirm CCl_4-induced chronic hepatitis. At d_0, d_{28}, d_{56} and d_{84} of the process, the portal perfused velocities (μL/min) in isolated rat livers were exactly controlled with a quantified pump. The pressure (mmHg) was monitored with a Physiological System. The geometric concentrations of phenylephrine or acetylcholine were added to a fixed volume (300 mL) of the circulating perfusate. The equation, the median effective concentration and its 95% confidence intervals of phenylephrine or acetylcholine were regressed with Prism-4 software in non-linear fit and various slopes. In the isolated perfused rat livers with chronic hepatitis, both median effective concentrations were defined as the pharmacodynamic model of portal hypertension.

RESULTS: At d_0, d_{28}, d_{56} and d_{84}, the equations of portal pressure potency from the concentrations of phenylephrine used to constrict the portal vein in isolated perfused rat livers were $Y = 0.1732 + 0.3970/[1 + 10^{(-4.3061-0.4407 X)}]$, $Y = -0.004934 + 0.12113/[1 + 10^{(-3.1247-0.3262 X)}]$, $Y = 0.0104 + 0.2643/[1 + 10^{(-8.8462-0.9579 X)}]$, and $Y = 0.01603 + 0.12107/[1 + 10^{(-5.1134-0.563 X)}]$; the median effective concentrations were 1.69×10^{-10} mol/L, 2.64×10^{-10} mol/L, 5.82×10^{-10} mol/L, and 8.24×10^{-10} mol/L, respectively. The equations from the concentrations of acetylcholine used to relax the portal vein were $Y = -0.4548 + 0.3274/[1 + 10^{(6.1538 + 0.5554 X)}]$, $Y = -0.05391 + 0.06424/[1 + 10^{(3.8541 + 0.3469 X)}]$, $Y = -0.2733 + 0.22978/[1 + 10^{(3.0472 + 0.3008 X)}]$, and $Y = -0.0559 + 0.053178/[1 + 10^{(5.6336 + 0.5883 X)}]$; the median effective concentrations were 8.40×10^{-10} mol/L, 7.73×10^{-12} mol/L, 5.98×10^{-11} mol/L, and 2.66×10^{-10} mol/L, respectively.

CONCLUSION: A pharmacodynamic model of portal hypertension in isolated perfused rat livers with chronic hepatitis was defined as the median effective concentrations of phenylephrine and acetylcholine.

Key words: Chronic hepatitis; Isolated portal perfused rat liver; Pharmacodynamic model; Portal hypertension

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INTRODUCTION

Patients with portal hypertension have significant mortality[1]. A lack of drugs[2] to treat this disease is derived from the failure to use the reversible mechanisms in its pathogenesis[3]. Being similar to amiloride, a candidate drug for portal hypertension[4], molecules from medical plants have been demonstrated to affect portal hypertension in rats[5-7] and in patients[8] with chronic hepatitis. The effect of these molecules on relaxation of the extrahepatic portal rings did not account for the efficacy of these therapies *in vivo*[9]. A novel mode of portal perfusion has been characterized with both controlled velocity and monitored pressure in the isolated portal perfused rat liver (IPPRL)[10-12]. With the primary velocity and preload at the various advanced stages of CCl_4-induced chronic hepatitis in rats, constriction with phenylephrine (PE) and relaxation with acetylcholine (Ach) were more sensitive than those reported previously[13,14]. With standardization of the IPPRL[15], both median effective concentrations of Ach and PE were defined as the pharmacodynamic model of portal hypertension. Both the controlled velocity and monitored pressure made the model sensitive enough for the basis of systems biology in portal regulation[16].

MATERIALS AND METHODS

Materials

Thirty two healthy male Wistar rats weighing 200-220 g were supplied by the Animal Center of the Chinese Academy of Medical Sciences. Standard rodent pellets for rats were prepared by Beijing Scientific Animal Feedstuff Company. The study was approved by the Animal Study Committee of the Chinese Academy of Medical Sciences. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China. All rats were maintained in a temperature-controlled room (25.0 ℃ ± 0.2 ℃) in the SPF laboratory, with a 12-h/12-h light/dark photoperiod and 45% ± 2% humidity. The rats were fed standard rodent pellets and allowed free access to tap water throughout the experiment.

Carbon tetrachloride (CCl_4, MW 153.84, CAS 56-23-5), Olive oil (CAS 8001-25-0) and Heparin sodium (MW 12 000, CAS 9041-08-1) were purchased from Sinopharm Chemical Reagent Company to induce chronic hepatic hepatitis or for anticoagulation.

As the perfusate in portal perfusion, Krebs-Henseleit solution consisted of KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaCl 118, and Glucose 11.0 mmol/L in the final concentration at pH 7.35-7.45 equilibrated with 95% O_2-5% CO_2 and warmed to 37.8 ℃ before being added to the livers.

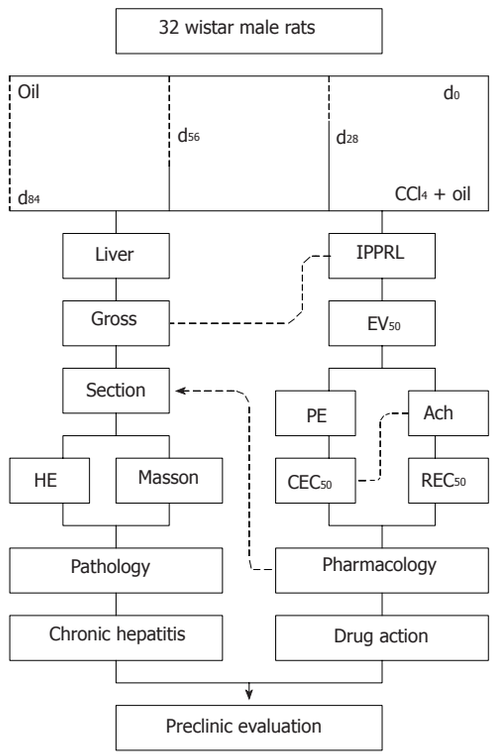

Figure 1 Group design of pharmacological model of portal hypertension. Oil indicated was administered subcutaneously with 3 mL/kg olive oil twice weekly for 84 d, as was CCl_4 + Oil 40% (v/v) CCl_4 in olive oil; IPPRL: Isolated portal perfused rat livers; EV_{50}: Median effective velocity; PE: Phenylephrine; Ach: Acetylcholine; CEC_{50}: Median effective concentration of PE constriction; REC_{50}: Median effective concentration of Ach relaxation; HE: Hematoxylin and eosin.

Phenylephrine hydrochloride (PE, MW 203.67, CAS 61-76-7) and Acetylcholine chloride (Ach, MW 181.67, CAS 60-31-1) were purchased from Sigma (United States) as the α_1-adrenoceptor and M_3-cholinoceptor agonist, respectively, used to elevate or reduce portal pressure.

Experimental design

Male Wistar rats were randomly divided into four groups. In four rats, PE was used to constrict the portal vein and in the other rats Ach was used to relax the portal vein in each group. Group 1 was the vehicle control without CCl_4 In this group, rats were subcutaneously administered 3 mL/kg olive oil twice weekly for 84 d. Groups 2, 3 and 4 were model groups with CCl_4-induced chronic hepatitis, the rats in these groups were subcutaneously administered the same volume of a mixture of 40% (v/v) CCl_4 in olive oil twice weekly for 28 d, 56 d and 84 d, beginning at d_{58}, d_{28}, and d_0, respectively (Figure 1). Forty-eight hours after the last CCl_4 injection, rats were anesthetized with 50 mg/kg pentobarbital sodium subcutaneously; a midline incision was made to expose the liver and its vessels. The hepatic artery, portal vein and hepatic vein were canalized. The remaining blood in the IPPRLs

Table 1 Hepatic pathological changes in model rats (mean ± SE, n_1 = 8)

Advanced	Lobule ratio	Collagen ratio
d_0	0.38 ± 0.05	0.0000700 ± 0.0001180
d_{28}	0.33 ± 0.04[b]	0.0019658 ± 0.0024864[b]
d_{56}	0.11 ± 0.04[b,d]	0.0043315 ± 0.0048768[b,d]
d_{84}	0.06 ± 0.01[b,d,f]	0.0143996 ± 0.0143860[b,d,f]

Lobule ratio is the average percentage obtained from ten random fields using Image-Pro Plus v 5.1 software, and each field had a ratio of lobule area per total analyzed field area in hematoxylin-eosin stained sections under a Digital Pathology System at × 20 magnification from isolated portal perfused rat livers. Collagen ratio is the average percentage obtained from ten random fields using Image-Pro Plus v 5.1 software, and each field had a ratio of collagen area per total analyzed field area in Masson-stained sections under a Digital Pathology System at × 40 magnification from isolated portal perfused rat livers. a and b denote significant ($^bP < 0.01$) differences between those at d_0 and at d_{28}, d_{56}, or d_{84}. c and d denote significant ($^cP < 0.05$ and $^dP < 0.01$) differences at d_{28} and at d_{56}, or d_{84}. e and f denote significant ($^eP < 0.05$ and $^fP < 0.01$) differences at d_{56} and at d_{84}.

was eliminated using Krebs-Henseleit perfusate through the hepatic artery. When portal perfusion was complete, a small portion of the liver was removed for pathological examination following fixation with 40 g/L formaldehyde solution and subsequent embedding in paraffin.

Protocol for portal perfusion

When CCl_4-induced chronic hepatitis was complete, eight rats from each group were randomized into two subgroups, one for PE constriction and the other for Ach relaxation of the portal vein. Each IPPRL was instrumented for portal pressure measurement.

Each IPPRL was perfused in a recirculation at a fixed temperature of 37.8 ℃ and equilibrated with 95% O_2-5% CO_2 mixed gas (Beijing Specialized Mixed Gas Institute), portal velocity was precisely controlled by a quantified BTO_1 pump (Beijing Yidaxk Technical Company), and 3935.50, 4720.63, 4753.35, and 5164.16 (μL/min) at d_0, d_{28}, d_{56}, and d_{84}, were chosen, respectively, the equation of portal perfusion median velocity (Y) from the day (x) of chronic hepatitis was Y = 13.28x + 4085 (r = 0.935, P < 0.01)[12].

The portal pressure of perfusion (mmHg) was continuously monitored and recorded with a strain-gauge transducer connected to the portal inflow cannula 6 cm proximal to the perfusion cannula with BL-420S Physiological Systems (Taimeng Instruments, Chengdu) according to a previously published method[10-14]. The global viability of livers was assessed by gross appearance and perfusate stable pH.

Pharmacodynamic actions

Perfusions were performed in the recirculating system containing 300 mL Krebs-Henseleit solution. Each preparation was allowed to stabilize for 15 min. The flow rate during each individual perfusion was maintained at a constant rate equalized to the portal perfusion median velocity at d_0, d_{28}, d_{56}, and d_{84}, respectively, the average portal pressure during this condition had been designated as the baseline. With a fixed volume of the recirculating perfusate in the portal perfusion system, cumulative geometric concentrations of PE (10^{-12}-10^{-6} mol/L) were added to elevate portal pressure.

After the median effective concentration of PE to constrict the portal vein was added, cumulative geometric concentrations of Ach (10^{-13}-10^{-7} mol/L) were added to reduce portal pressure.

Concentration-response curves were obtained following the addition of PE and Ach, and the changes in intrahepatic resistance expressed as the percentage increase or decrease in perfusion pressure from baseline in the various portal perfused velocities were obtained.

Pathological changes due to chronic hepatitis

To observe pathological changes after portal perfusion, a portion of the left liver lobe (40 mg) from each liver was fixed in 40 g/L formaldehyde solution for 48 h, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin-eosin and Masson according to standard procedures.

Images were acquired with a Nano Zoomer Digital Pathology system (Hamamatsu, Japan), at a low magnification (× 20); all the compartments of the liver were analyzed. At high magnification (× 40), the collagen density in the liver section was quantified using a computerized image analysis system (Image-Pro Plus v 5.1). The density of collagen in blinded specimens was expressed as a percentage (the ratio of collagen area per total analyzed field area). The average of the score taken from ten random fields was used to generate a single score for each IPPRL.

Statistical analysis

All primary data are presented as means ± SE for each dosage in each group. Statistical significance was calculated using Student's t test between groups, P < 0.05 was significant. (1) Dose-effect relationship: The equation, the median effective concentration and its 95% confidence intervals of PE or Ach were calculated by regression analysis using Graph-Pad Prism 4 in non-linear fit and various slopes, to express the dose-effect relationship; and (2) Time-effect relationship: The median effective concentrations of PE or Ach were calculated by linear regression analysis with the duration (0 d, 28 d, 56 d, and 84 d) of chronic hepatitis, to express the time-effect relationship of pathological conditions affecting the portal response to both molecules.

RESULTS

Pathological changes due to chronic hepatitis

Lobule or pseudo-lobule ratio: Hepatic tissues were clear in hematoxylin and eosin-stained sections. When compared with those in control rats at d_0, the lobule ratios (Table 1) in the model rats at d_{28}, d_{56} and d_{84} were significantly decreased by 4.04%, 70.22%, and 83.82%, respectively (P < 0.01). When compared with those at d_{28}, the pseudo-lobule ratios in the model rats at d_{56} and d_{84} were significantly decreased by 65.35% and 81.00%, respectively (P < 0.01). In addition,

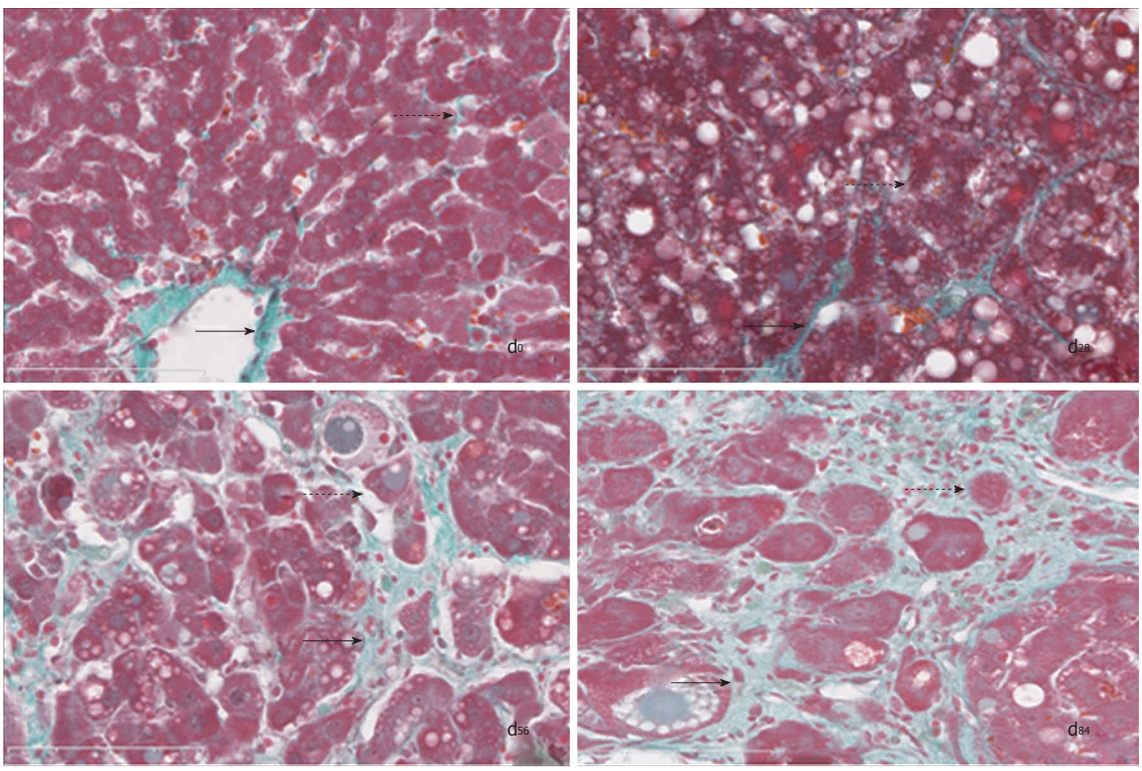

Figure 2 Collagen distributions in rat model livers (Masson × 40). Normal hepatic structure (d_0) was demonstrated in control rats administered 3 mL/kg olive oil subcutaneously twice weekly for 84 d. Hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}) were seen in the chronic hepatitis model rats administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice weekly for 28 d, 56 d, and 84 d, respectively. There was less collagen mainly around the central veins (solid arrow) and the portal areas, with some staining noise signals in lobules (dashed arrow) in normal hepatic structure (d_0). The collagen fiber bundles (solid arrow) had partly spread into the lobules from the portal areas, the hepatic cells had obvious watery and fatty degeneration (dashed arrow) and had not been isolated by collagen during hepatic degeneration (d_{28}). The collagen fiber bundles (solid arrow) had completely separated some of the lobules to form many pseudo-lobules, several hepatic cells with obvious fatty degeneration (dashed arrow) had been completely isolated by collagen in hepatic fibrosis (d_{56}). The collagen fiber bundles (solid arrow) had limited smaller pseudo-lobules, some single hepatic cells (dashed arrow) were completely isolated by collagen in hepatic cirrhosis (d_{84}).

these ratios were significantly decreased by 45.67% at d_{84} compared with those at d_{56} ($P < 0.01$).

Hepatic collagen distribution: Hepatic histological changes in Masson-stained sections showed collagen depositions along with CCl_4-induced chronic hepatitis (Figure 2). (1) Normal structure at d_0: The histological structure in control rats showed normal hepatic architecture with some fatty degeneration and less collagen located at the lobules; (2) Degeneration at d_{28}: The pathological changes in the model rats at d_{28} showed mainly hepatic fatty degeneration and cellular swelling, collagen was deposited around the center veins, thus the enlarged hepatic cords severely narrowed the hepatic sinusoid; (3) Hepatic fibrosis at d_{56}: The pathological changes in the model rats at d_{56} showed more collagen deposited in the lobules, thus the enlarged hepatic cords led to significant widening of the hepatic sinusoid; and the collagen in interlobular area extended into the lobules, some separating the lobules completely, therefore the direction of the circulating blood did not change in the hepatic sinusoid of the lobules; and (4) Hepatic cirrhosis at d_{84}: The pathological changes in the model rats at d_{84} showed extensive collagen deposited in the lobules, which were all pseudo-lobules instead of normal lobules, thus the direction of the circulating blood had completely changed in the hepatic sinusoid.

Deposited collagen ratio: Compared with the control rats (Table 1), the collagen ratio in the model rats at 28 d, 56 d and 84 d was significantly increased by 2707.65%, 60 860.51%, and 20 466.49%, respectively ($P < 0.01$). Compared with the model rats at 28 d, the collagen ratio in the model rats at 56 d and 84 d was significantly increased by 120.34% and 632.52%, respectively ($P < 0.01$). The collagen ratio in the model rats at 84 d increased by 232.44% compared to that at 56 d ($P < 0.01$).

Pharmacodynamic actions on the portal vein

Phenylephrine elevated portal pressure: Geometric concentrations of PE to activate the α_1 receptor were added to the recirculating perfusate to elevate perfused portal pressure (Table 2 and Figure 3). The equation, the median effective concentration of PE and its 95% confidence intervals were regressed: (1) dose-effect at d_0: The data showed that the equation of PE was $Y = 0.1732 + 0.3970/[1 + 10^{(-4.3061-0.4407\ x)}]$ ($r = 0.9701$, $P < 0.01$); the median effective concentration with its 95% confidence intervals was 1.69×10^{-10} (4.9769×10^{-12} - 5.7599×10^{-9}) mol/L; (2) dose-effect at d_{28}: The data showed that the equation of PE was $Y = -0.004934 + 0.121134/[1 +$

Table 2 Phenylephrine elevates portal pressure (mean ± SE, n_i = 4)

Log [PE (mol/L)]	d_0	d_{28}	d_{56}	d_{84}
-12	0.210 ± 0.19	0.013 ± 0.02	0.013 ± 0.02	0.019 ± 0.03
-11	0.260 ± 0.16	0.025 ± 0.03	0.017 ± 0.02	0.024 ± 0.04
-10	0.360 ± 0.18	0.044 ± 0.06	0.046 ± 0.06	0.047 ± 0.07
-9	0.420 ± 0.24	0.072 ± 0.09	0.182 ± 0.25	0.076 ± 0.09
-8	0.550 ± 0.37	0.090 ± 0.12	0.247 ± 0.27	0.119 ± 0.10
-7	0.520 ± 0.37	0.093 ± 0.12	0.269 ± 0.27	0.123 ± 0.11
-6	0.570 ± 0.24	0.113 ± 0.13	0.286 ± 0.28	0.138 ± 0.12

Primary data were used to calculate the dose-effect relationship between phenylephrine and portal vein constriction in the isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. PE: Phenylephrine.

Table 3 Acetylcholine reduces portal pressure (mean ± SE, n_i = 4)

Log [Ach (mol/L)]	d_0	d_{28}	d_{56}	d_{84}
-13	-0.16 ± 0.12	-0.002 ± 0.01	-0.076 ± 0.08	-0.001 ± 0.01
-12	-0.19 ± 0.10	-0.009 ± 0.01	-0.091 ± 0.10	-0.006 ± 0.01
-11	-0.31 ± 0.07	-0.025 ± 0.01	-0.125 ± 0.15	-0.012 ± 0.01
-10	-0.39 ± 0.08	-0.035 ± 0.18	-0.176 ± 0.23	-0.019 ± 0.01
-9	-0.42 ± 0.08	-0.043 ± 0.04	-0.203 ± 0.26	-0.041 ± 0.02
-8	-0.44 ± 0.12	-0.049 ± 0.05	-0.225 ± 0.26	-0.050 ± 0.03
-7	-0.47 ± 0.14	-0.052 ± 0.07	-0.256 ± 0.28	-0.054 ± 0.03

Primary data were used to calculate the dose-effect relationship between acetylcholine and portal vein relaxation in the isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. Ach: Acetylcholine.

$10^{(-3.1247-0.3262\ x)}$] (r = 0.9937, P < 0.01); the median effective concentration with its 95% confidence intervals was 2.64×10^{-10} (7.1864×10^{-12} - 9.6834×10^{-9}) mol/L; (3) dose-effect at d_{56}: The data showed that the equation of PE was $Y = 0.0104 + 0.2643/[1 + 10^{(-8.8462-0.9579\ x)}]$ (r = 0.9980, P < 0.01); the median effective concentration with its 95% confidence intervals was 5.82×10^{-10} (3.0691×10^{-10} - 1.1031×10^{-9}) mol/L; (4) dose-effect at d_{84}: The data showed that the equation of PE was $Y = 0.01603 + 0.12107/[1 + 10^{(-5.1134-0.563\ x)}]$ (r = 0.9963, P < 0.01); the median effective concentration with its 95% confidence intervals was 8.24×10^{-10} (2.2476×10^{-10} - 3.0207×10^{-9}) mol/L; and (5) time-effect: The linear regression equation was $Y = 0.081\ x + 1.173$ (r = 0.981, P < 0.01) between the median effective concentrations of PE (1.69, 2.64, 5.82 and 8.24) $\times 10^{-10}$ mol/L and the durations (0 d, 28 d, 56 d and 84 d) of chronic hepatic hepatitis.

Acetylcholine reduced portal pressure: Geometric concentrations of Ach to activate the M_3 receptor were added to the circulating perfusate to reduce perfused portal pressure. The equation, the median effective concentration of Ach and its 95% confidence intervals of Ach were regressed (Table 3 and Figure 4): (1) dose-effect at d_0: The data showed that the equation of Ach was $Y = -0.4548 + 0.3274/[1 + 10^{(6.1538\ +\ 0.5554\ x)}]$ (r = 0.9950, P < 0.01); the median effective concentration with its 95% confidence intervals was 8.40×10^{-10} (1.3263×10^{-12} - 5.3240×10^{-11}) mol/L; (2) dose-effect at d_{28}: The data showed that the equation of Ach was $Y = -0.05391 + 0.06424/[1 + 10^{(3.8541\ +\ 0.3469\ x)}]$ (r = 0.9982, P < 0.01); the median effective concentration with its 95% confidence intervals was 7.73×10^{-12} (7.3614×10^{-13} - 8.1095 ×

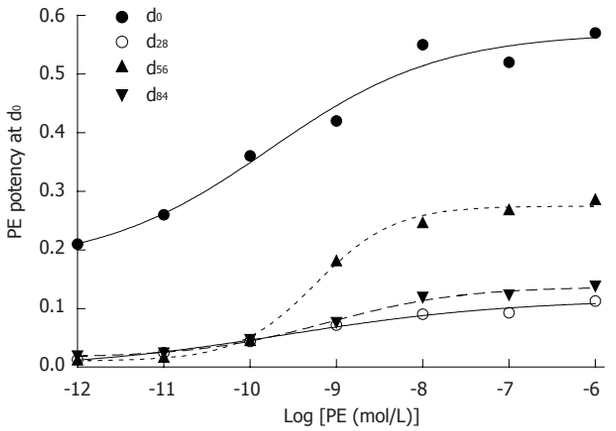

Figure 3 Phenylephrine elevates portal pressure (mean ± SE, n_i = 4). The dose-effect relationship between phenylephrine and portal vein constriction in isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. PE: Phenylephrine.

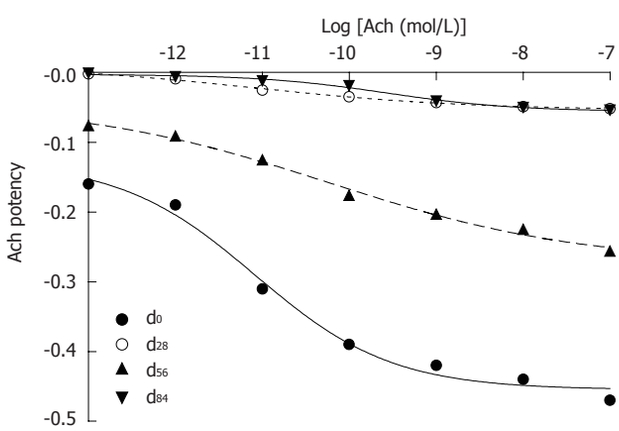

Figure 4 Acetylcholine reduces portal pressure (mean ± SE, n_i = 4). The dose-effect relationship between Acetylcholine and portal vein relaxation in isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. Ach: Acetylcholine.

10^{-11}) mol/L; (3) dose-effect at d_{56}: The data showed that the equation of Ach was Y = -0.2733 + 0.22978/[1 + $10^{(3.0472 + 0.3008\ x)}$] ($r$ = 0.9964, P < 0.01); the median effective concentration with its 95% confidence intervals was 5.98 × 10^{-11} (4.2797 × 10^{-12} - 8.3556 × 10^{-11}) mol/L; (4) dose-effect at d_{84}: The data showed that the equation of Ach was Y = -0.0559 + 0.053178/[1 + $10^{(5.6336 + 0.5883\ x)}$] ($r$ = 0.9956, P < 0.01); the median effective concentration with its 95% confidence intervals was 2.66 × 10^{-10} (6.5887 × 10^{-11} - 1.0701 × 10^{-9}) mol/L; and (5) time-effect: The linear regression equation was Y = 0.046 X -1.470 (r = 0.945, P < 0.05) between the median effective concentrations of Ach (0.0773, 0.598 and 2.66) × 10^{-10} mol/L and the durations (28 d, 56 d and 84 d) of chronic hepatitis.

DISCUSSION

Patients with portal hypertension have significant morbidity and mortality[1] without special drugs[2] based on the reversible pathogenesis of this disease[3]. Some candidate drugs from chemicals and medical plants have demonstrated effects on portal hypertension in animal experiments and in clinical trials[4-8]. Data from the extrahepatic portal rings failed to account for these effects[9]. Consequently, sensitive portal perfusion for intra-hepatic portal resistance has been developed with both controlled velocity and monitored pressure in IPPRLs[10-14]. The pharmacodynamic model of portal hypertension has further been defined as the median effective concentrations of Ach and PE in the IPPRLs at various stages of CCl_4-induced chronic hepatitis.

At d_0, d_{28}, d_{56}, and d_{84} in CCl_4-induced chronic hepatitis, there were similar portal pressure potency equations with various coefficients due to the concentrations of PE and Ach in the IPPRLs. The median effective concentrations of PE increased geometrically during the process, suggesting that the function of portal smooth muscle cells gradually decreased. A similar effect was noted with the median effective concentrations of Ach in advanced stages, which suggested that portal endothelia were gradually damaged. During portal perfusion with both controlled pressure and monitored velocity, as reported previously, the effective range of PE and Ach concentrations was from 10^{-3} mol/L to 10^{-8} mol/L[15,16]. In this novel model of portal perfusion with both controlled velocity and monitored pressure, the effective range was from 10^{-6} mol/L to 10^{-12} mol/L, which indicated that this novel mode was more sensitive than the previous mode by 10^3-10^6 times in IPPRLs.

Hepatocyte injuries originate from the free radicals of CCl_4 metabolites[3]. Amiloride reduced intra-hepatic portal resistance through inhibition of the Rho kinase pathway in hepatic stellate cells[4]. Glycyrrhizinate and Salvianolic acid B are representative molecules from medical plants used for portal hypertension in rats[5-7] and patients[8] with chronic hepatitis, however, their biomolecular mechanisms are not yet clear. PE, as a α_1-adrenoceptor agonist, constricts vascular smooth muscle[13] and Ach, as a M_3-cholinoceptor agonist in endothelia, relaxes vascular smooth muscle[14]. Due to these mechanisms in IPPRLs, the median perfused velocity in portal pressure has been defined as the primary flow rate of portal perfusion in this novel mode, the median effective concentration of PE for elevating portal pressure as the preload, the median effective concentration of Ach for reducing the portal pressure as the positive action at the classic stages of the pathological process in chronic hepatitis.

The pharmacodynamic model of portal hypertension has been defined as both the median effective concentrations of PE and Ach in the IPPRLs with advanced chronic hepatitis in this study. This model may be used to evaluate the preclinical effects of candidate drugs for the treatment of portal hypertension. Both controlled velocity and monitored pressure[10-12] made this model more sensitive than previous models[13-14]. Based on the standardization[15] of the IPPRL, this sensitive model is considered the basis of systems biology for portal regulation in an isolated setting[16].

COMMENTS

Background

Portal hypertension results in significant mortality without the administration of special drugs. Candidate drugs for this condition require serious pre-clinical evaluation using suitable methods.

Research frontiers

The recently identified reversible pathogenesis of portal hypertension may allow the development of new drugs for this disease. Candidate drugs derived from medical plants used in Chinese medical practices have confirmed its reversible pathogenesis. A sensitive pressure transducer was used here for exploiting the reversible pathogenesis as the pharmacological models. The optimal conditions for each step of the procedure can be defined as an available model.

Innovations and breakthroughs

Reversible portal hypertension was replicated in the advanced stages of chronic hepatitis in rats using CCl_4. A pharmacological model was developed using the median primary velocity of perfused flow as the anatomical preload, median effective concentrations of phenylephrine to constrict portal veins as the physiological preload, and the median effective concentrations of acetylcholine to relax portal veins in IPPRLs with chronic hepatitis.

Applications

This novel pharmacological model can be used to evaluate candidate drugs for the treatment of portal hypertension.

Terminology

This novel mode of portal perfusion is characterized by both controlled velocity and monitored pressure in the isolated portal perfused rat livers. The controlled velocity creates the optimal conditions for research purposes, and the monitored pressure gives exact data from vascular smooth muscle or endothelia.

Peer review

The authors investigated to develop a pharmacodynamic model for portal hypertension from chronic hepatitis. They have developed a pharmacodynamic model for portal hypertension in rats with chronic hepatitis and demonstrated that the model had been defined as the median effective concentrations of phenylephrine and acetylcholine. The results are clear and informative for the study on portal hypertension.

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BRIEF ARTICLE

Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences

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Abstract

AIM: To assess the safety of *Bifidobacterium longum* (*B. longum*) JDM301 based on complete genome sequences.

METHODS: The complete genome sequences of JDM301 were determined using the GS 20 system. Putative virulence factors, putative antibiotic resistance genes and genes encoding enzymes responsible for harmful metabolites were identified by blast with virulence factors database, antibiotic resistance genes database and genes associated with harmful metabolites in previous reports. Minimum inhibitory concentration of 16 common antimicrobial agents was evaluated by *E*-test.

RESULTS: JDM301 was shown to contain 36 genes associated with antibiotic resistance, 5 enzymes related to harmful metabolites and 162 nonspecific virulence factors mainly associated with transcriptional regulation, adhesion, sugar and amino acid transport. *B. longum* JDM301 was intrinsically resistant to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptible to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and trimethoprim-sulphamethoxazol. JDM301 was moderately resistant to bacitracin, while an earlier study showed that bifidobacteria were susceptible to this antibiotic. A tetracycline resistance gene with the risk of transfer was found in JDM301, which needs to be experimentally validated.

CONCLUSION: The safety assessment of JDM301 using information derived from complete bacterial genome will contribute to a wider and deeper insight into the safety of probiotic bacteria.

Key words: *Bifidobacterium longum*; Safety assessment; Genome; Antibiotic resistance; Harmful metabolite; Virulence factor

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INTRODUCTION

Bifidobacteria spp are high-GC content, Gram-positive bacteria which belong to the *Actinobacteria* branch and these species naturally colonize the gastrointestinal tract (GIT) of mammals, birds and insects[1]. Scientists have determined the major probiotic properties of *Bifidobacteria* spp isolated from the human intestine and these properties include the strengthening of the intestinal barrier, modulation of the immune response and antagonism of pathogens[2].

Bifidobacterium spp has been reported to possess various glycosyl hydrolases (GH) and these hydrolases metabolize plant- or milk-derived oligosaccharides including nondigestible ones such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS)[3,4]. The capability to utilize nondigestible oligosaccharides confers a competitive advantage to *Bifidobacterium* spp in the human gut.

Bifidobacterium longum (*B. longum*) and various other bifidobacteria strains are often added to probiotic products in combination with other lactic acid bacteria (LAB). Through their long and safe history of application, LAB have acquired the status of "Generally Regarded As Safe" (GRAS), but the safety of *Bifidobacteria* and other LAB strains selected for probiotics still need to be carefully evaluated. The key safety aspects for use of bifidobacteria and other LAB strains in probiotics include antibiotic resistance, production of harmful metabolites and the potential for virulence. Antibiotic resistance in potential probiotic strains is not considered a risk factor unless resistance is transferred to pathogens or it renders the *probiotic untreatable* in very rare cases of infection[5]. Biogenic amines, D-lactic acid, azoreductases and nitroreductases produced by bifidobacteria and other LAB strains are potential health hazards[6,7] and the safety of some of these compounds have been evaluated[8]. Virulence genes may be present in commensal bacteria and absence of virulence in these bacteria needs to be proved on a case by case basis.

Probiotic agents are widely used in the food and drug industry and as more commercial probiotic products are being introduced in the market, it is timely to reassess the safety of these probiotic products using the latest technology. Information from the complete genome sequences of *Bifidobacteria* will provide additional insight into the genetic basis for their safety. We sequenced the complete genome sequences of *B. longum* JDM301 (GenBank accession number CP002010), a commercial strain used widely in China with several probiotic functions, for this purpose[9].

The aim of the present work was to assess the safety of *B. longum* JDM301 based on complete genome sequences. The criteria used were the potential to transfer antibiotic resistance to pathogens, the potential for production of harmful metabolites and the potential for virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions

JDM301 was isolated from commercial probiotic product and identified using a sequence analysis of its 16S rRNA gene. De Man-Rogosa-Sharpe (MRS) broth (Difco) supplemented with 0.05% L-cysteine·HCl (Sigma) was used for cultivating JDM301. Cultures were incubated at 37 ℃ under anaerobic conditions.

Genome sequencing and assembly

We determined the complete genome sequence of JDM301 at the Chinese National Human Genome Center in Shanghai using the GS 20 system (454 Life Science Corporation, Branford, Connecticut). A total of 192 888 reads with an average length of 210 bps were assembled into 112 contigs by the 454 assembly tool. The order of most large contigs, which were larger than 500 bp, was determined through the basic local alignment search tool (BLAST) analysis with the reference strain *B. longum* ATCC15697 (GenBank accession number CP001095) and the others were arranged by multiplex polymerase chain reaction (PCR). Gap closure was carried out by sequencing gap-spanning PCR products or clones using ABI 3730 xl DNA sequencers. Primer design and sequence assembly were performed by the Phred/Phrap/Consed software package[9]. The locations of low-quality sequences in genome were verified by directly resequencing the PCR products spanning the low-quality sequences using the ABI 3730 xl DNA sequencers.

Statistical analysis

The genome sequences of *Bifidobacteria* except JDM301 were retrieved from GenBank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)[10]. Potential open reading frames (ORF) were identified using Glimmer[11] and ZCURVE[12] 1.0 using default settings. Clusters of orthologous group (COG) functional categories were used for functional classification of all genes in the genome sequences of JDM301 and the COGs. A BLAST analysis of the translations with GenBank's nonredundant database was performed, which was followed by manual curation. The best matches were chosen for preliminary product assignments. Insertion sequences (IS) elements, prophage sequences and clustered regularly interspaced short palindromic repeats (CRISPR) were identified by IS finder (http://www-is.biotoul.fr/is.html), Prophage Finder[13] and CRISPRFinder (http://crispr.u-psud.fr/crispr/)[14] respectively. Putative orthologues were determined by Omics Explorer (http://omics.biosino.org:14000/kweb/about.jsp) using default values. Ribosomal RNA genes were detected on the basis of BLASTN searches and transfer RNA genes were identified using tRNAscan-SE[15]. The atlas of genome was drawn using GenomeViz1.1[16]. Putative virulence factors and putative antibiotic resistance genes were identified by blast with virulence factors database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm)[17] and antibiotic resistance genes database (ARDB) (http://ardb.cbcb.umd.edu/)[18] respectively.

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) of 16 common

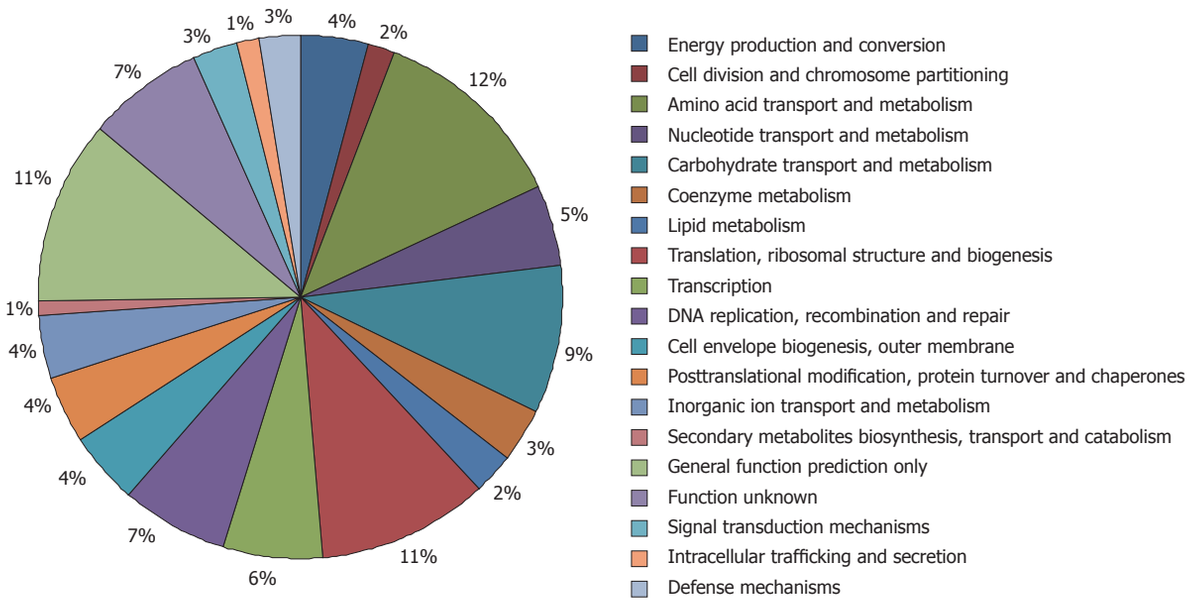

Figure 1 Functional distribution of *Bifidobacterium longum* core proteins. A total of 1265 proteins were conserved in all four *Bifidobacterium longum* (*B. longum*) strains (*B. longum* JDM301, *B. longum* NCC2705, *B. longum* DJO10A and *B. longum* ATCC15697), representing the "core" genome of *B. longum*.

antimicrobial agents was evaluated by *E*-test (AB Biodisk, Solna, Sweden) including amoxicillin (0.016-256 mg/L), amikacin (0.016-256 mg/L), ampicillin (0.016-256 mg/L), bacitracin (0.016-256 mg/L), cephalothin (0.016-256 mg/L), ciprofloxacin (0.002-32 mg/L), cefotaxime (0.016-256 mg/L), chloramphenicol (0.016-256 mg/L), erythromycin (0.016-256 mg/L), gentamicin (0.016-256 mg/L), imipenem (0.002-32 mg/L), rifampicin (0.016-256 mg/L), streptomycin (0.016-256 mg/L), tetracycline (0.016-256 mg/L), trimethoprim-sulphamethoxazol (0.002-32 mg/L), and vancomycin (0.016-256 mg/L). Tests were done with MRS agar supplemented with 0.05% L-cysteine·HCl (Sigma) and were conducted in triplicate for each antibiotics. Cultures sub-inoculated into the MRS agar supplemented with 0.05% L-cysteine·HCl were incubated anaerobically at 37 ℃ for 24 h.

RESULTS

Comparative genomic analysis of Bifidobacteria

The predicted proteins of *B. longum* JDM301 were functionally categorized. The functional distribution of genes assigned to clusters of orthologous groups of proteins was relatively similar to the other *Bifidobacteria*, e.g., *B. longum* and *B. adolescentis* in the GIT and *B. dentium* in the oral cavity[3,4,19]. The top four functional categories in *B. longum* JDM301, namely, carbohydrate transport and metabolism, amino acid transport and metabolism, were identical with other *Bifidobacteria*[20].

Putative orthologues among *B. longum* strains were determined in a comparative study (Figure 1). Overall, 1265 proteins were conserved in all four *B. longum* strains (*B. longum* JDM301, *B. longum* NCC2705, *B. longum* DJO10A and *B. longum* ATCC15697). These proteins represent the "core" genome of *B. longum*, whereas 219 proteins are unique to *B. longum* JDM301. The most common functional distributions of the core proteins were these involved in housekeeping functions including amino acid transport and metabolism, translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism and DNA replication, recombination and repair. Twenty-one percent of the core proteins were dedicated to carbohydrate and amino acid transport and metabolism, indicating the important roles of these proteins in *Bifidobacteria*.

Stability of the genome of B. longum JDM301

Horizontal gene transfer (HGT) events are responsible for introduction of alien genes, which may reinforce the adaptation of bacteria in their specific niches. Genes on plasmids, bacteriophages, genomic islands and IS are sensitive to HGT[21]. Twelve phage-related fragments were identified in the genome of *B. longum* JDM301[9], but no complete prophages were found. The JDM301 chromosome also possesses 15 complete or disrupted IS elements[9]. The number of IS element in JDM301 is relatively smaller than the other sequenced *B. longum* spp[3,4]. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system. No CRISPR was discovered in the genome.

One complete type Ⅱ restriction-modification (R-M) system and one type Ⅲ R-M system were present in the genome of JDM301. A complete and incomplete type Ⅰ R-M system was also identified in this genome. Two complete type Ⅱ R-M systems and one type Ⅰ R-M system were present in the genome of *B. longum* NCC2705, while one complete type Ⅱ R-M system and type Ⅰ R-M system were found in *B. longum* DJO10A.

Antibiotic resistance determinants

The antibiotic resistance genes in JDM301 were identified

Table 1 Putative antibiotic resistance genes identified in the genome of *Bifidobacterium longum* JDM301

Antibiotics	Antibiotic resistance genes	Product name
Bacitracin	*BLJ_1636*	ABC transporter-related protein
	BLJ_0984	ABC transporter-related protein
	BLJ_0923	ABC transporter-related protein
	BLJ_1055	Undecaprenyl pyrophosphate phosphatase
	BLJ_1119	Bacitracin transport ATP-binding protein bcrA
Vancomycin	*BLJ_0853*	VanU
	BLJ_1764	Dehydrogenase VanH
	BLJ_1084	Sensor protein vanSB
	BLJ_0707	VanSD5
	BLJ_0343	Histidine kinase VanSc3
	BLJ_0287	D-Ala: D-Lac ligase VanD
Multiple drugs	*BLJ_1090*	ATP-binding protein
	BLJ_1650	Lsa
	BLJ_1437	LmrB
	BLJ_0618	Multidrug export protein MepA
	BLJ_0769	Efflux transporter, RND family, MFP subunit
	BLJ_0181	Multidrug efflux protein QacB
	BLJ_1062	Multidrug export protein MepA
Chloramphenicol	*BLJ_1672*	Chloramphenicol resistance protein
	BLJ_1322	Chloramphenicol resistance protein
Thiostrepton	*BLJ_0885*	Thiostrepton-resistance methylase
Penicillin	*BLJ_1301*	Penicillin binding protein
Kasugamycin	*BLJ_2030*	S-adenosylmethionine-6-N', N'-adenosyl (rRNA) dimethyltransferase
Tetracycline	*BLJ_0814*	Tetracycline-resistance determinant tetV
	BLJ_1245	TetW
	BLJ_0594	Tetracycline resistance protein
	BLJ_1401	TetQ
Carbomycin	*BLJ_1625*	Carbomycin resistance protein
Sulfonamide	*BLJ_1629*	Dihydropteroate synthase
Tetracenomycin C	*BLJ_1624*	Tetracenomycin C efflux protein
Trimethoprim	*BLJ_1657*	dihydrofolate reductase
Macrolide	*BLJ_0925*	Macrolide-efflux protein
	BLJ_1936	Macrolide-efflux protein
	BLJ_0819	Macrolide-efflux protein
	BLJ_0042	Macrolide-efflux protein
	BLJ_1154	Macrolide-efflux protein variant

ABC: ATP-binding cassette; RND: Resistance-nodulation-cell division.

using ARDB (E < 1e-2, coverage > 70%)[18]. Homologs of the antibiotic resistance determinants for vancomycin, methicillin, tetracycline, chloramphenicol and trimethoprim were found in the genome of JDM301 (Table 1) and 6 putative resistance genes for vancomycin. *B. longum* JDM301 also possessed 5 putative bacitracin efflux pumps, 5 homologs of macrolide efflux proteins. Additionally, 7 putative multidrug resistance efflux pumps belonging to an ATP-binding cassette (ABC)-type transport system, a major facilitator superfamily transporter and resistance-nodulation-cell division (RND) family were found in the genome. The genome of *B. longum* JDM301 also contains 4 tetracycline resistance genes encoding for TetV, TetW, TetPB and TetQ. The gene for TetW shows a strong difference in G + C content (53.0%) compared to the average value of *B. longum* JDM301 (59.8%) genome and it is flanked by genes encoding for integrases, indicating that this region may have been acquired by HGT.

Table 2 Minimum inhibitory concentration values of 16 antibiotics for *Bifidobacterium longum* JDM301

Antibiotics	Minimum inhibitory concentration (mg/L)
Ciprofloxacin	> 32
Amikacin	> 256
Gentamicin	> 256
Bacitracin	26.67
Streptomycin	170.67
Vancomycin	0.9
Amoxicillin	0.064
Cephalothin	1.33
Chloramphenicol	0.25
Erythromycin	0.04
Ampicillin	0.058
Cefotaxime	0.19
Rifampicin	0.074
Tetracycline	8
Imipenem	0.19
Trimethoprim-sulphamethoxazol	1.83

The antibiotic susceptibility of *B. longum* JDM301 to 16 antibiotics was determined by an *E*-test to probe the *in silico* analyses of the complete genome sequence. The results of the *E*-test are summarized in Table 2. The breakpoints for determining susceptibility were determined using accepted protocols[22-25]. *B. longum* JDM301 showed a high resistance to ciprofloxacin, amikacin and gentamicin, moderate resistance to streptomycin and bacitracin and were sensitive to tetracycline, vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol.

Putative enzymes for harmful metabolites

Genes encoding enzymes responsible for harmful metabolites, including beta-glucosidase (GS), arylsulphatase (AS), beta-glucuronidase (GN), nitroreductase (NR), azoreductase (AR), D-lactate dehydrogenase (DLD), amino acid decarboxylase (AD) and conjugated bile salt hydrolase (CBSH) were searched for in the genome of *B. longum* JDM301. Two GS genes (BLJ_1280, BLJ_1540) and one CBSH gene (BLJ_0948) were found in the chromosome of *B. longum* JDM301. Homologs of DLD (BLJ_1306, BLJ_1436) and NR (BLJ_1980) were also discovered in the genome. Enzymes involved in putatively harmful metabolites, AR, GN, AD and AS were not found in JDM301 genome.

Putative virulence factors

Published reports of rare infections involving *Lactobacilli* or *Bifidobacteria* are available and the potential virulence of *Lactobacilli* or *Bifidobacteria* used as probiotics should be assessed[5]. Putative virulence genes of *B. longum* JDM301 were determined by BLAST analysis of the VFDB[17]. A total of 141 homologs of virulence factors were identified in the genome of JDM301, including 28 sugar-binding transcriptional regulators, 20 genes associated

Table 3 Putative virulence factors identified in the genome of *Bifidobacterium longum* JDM301

Query	Identity	Subject	Predicted functions
BLJ_1089	24.9	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_1835	26.36	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_0323	29.3	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_1476	22.11	VFG2378	6 kDa early secretory antigenic target esxA
BLJ_0992	32.98	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_1080	34.81	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_1968	37.3	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_0770	37.43	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_0026	35.71	VFG1404	ahpC
BLJ_0136	28.73	VFG2218	ATPase VirB11 homolog
BLJ_0880	24.18	VFG1042	ATP-binding protein FecE
BLJ_0787	47.92	VFG0077	ATP-dependent Clp protease proteolytic subunit
BLJ_0786	53.8	VFG0077	ATP-dependent Clp protease proteolytic subunit
BLJ_0948	37.66	VFG2162	Bile salt hydrolase
BLJ_1243	22.97	VFG2242	Conjugal transfer protein trag
BLJ_0551	26.54	VFG1108	Conserved hypothetical protein
BLJ_1951	29.85	VFG1269	Cyclolysin secretion ATP-binding protein
BLJ_1925	32.31	VFG1269	Cyclolysin secretion ATP-binding protein
BLJ_1863	45.5	VFG0079	Endopeptidase Clp ATP-binding chain C
BLJ_1465	56.77	VFG0079	Endopeptidase Clp ATP-binding chain C
BLJ_0713	30.12	VFG0925	Ferric enterobactin transport ATP-binding protein fepC
BLJ_1872	25.51	VFG2225	GDP-mannose 4,6-dehydratase
BLJ_1324	32.49	VFG1399	glnA1
BLJ_0624	62.11	VFG1399	glnA1
BLJ_1834	29.47	VFG0313	Glucose/galactose transporter
BLJ_1926	30.02	VFG1557	HlyB protein
BLJ_1477	56.12	VFG1855	Hsp60, 60K heat shock protein HtpB
BLJ_0064	26.21	VFG1397	hspX
BLJ_1444	40.85	VFG1563	Hypothetical protein
BLJ_1606	27.78	VFG1593	Hypothetical protein
BLJ_1640	30.81	VFG1593	Hypothetical protein
BLJ_0011	22.16	VFG1604	Hypothetical protein
BLJ_1513	26.3	VFG1604	Hypothetical protein
BLJ_1846	27.67	VFG1604	Hypothetical protein
BLJ_0337	44.25	VFG1630	Hypothetical protein
BLJ_0336	44.38	VFG1630	Hypothetical protein
BLJ_1500	23.53	VFG1963	Hypothetical protein Cj1435c
BLJ_1169	24.64	VFG1390	Hypothetical protein Rv0981
BLJ_0708	36.8	VFG1390	Hypothetical protein Rv0981
BLJ_0802	28.83	VFG1824	Hypothetical protein Rv3133c
BLJ_1357	30.46	VFG1824	Hypothetical protein Rv3133c
BLJ_1113	32.41	VFG1824	Hypothetical protein Rv3133c
BLJ_0835	32.42	VFG1824	Hypothetical protein Rv3133c
BLJ_0859	27.93	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_0348	28.13	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_0530	29.29	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_2016	35.81	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_1875	36.19	VFG1627	IS100 transposase; transposase ORFA
BLJ_1249	37.55	VFG1627	IS100 transposase; transposase ORFA
BLJ_1252	39.22	VFG1627	IS100 transposase; transposase ORFA
BLJ_0930	42.29	VFG1627	IS100 transposase; transposase ORFA
BLJ_1966	30.68	VFG1485	L7045
BLJ_1850	59.7	VFG1411	leuD
BLJ_0379	39.24	VFG0320	Lipopolysaccharide core biosynthesis protein (kdtB)
BLJ_1549	22.02	VFG1817	mbtA
BLJ_1204	25.8	VFG0574	Mg<up>2+</up> transport protein
BLJ_2010	30.62	VFG0574	Mg<up>2+</up> transport protein
BLJ_1270	28.62	VFG1116	N-acetylglucosamine-6-phosphate deacetylase
BLJ_1832	21.89	VFG1109	N-acetylneuraminate lyase, putative
BLJ_0490	25.95	VFG1109	N-acetylneuraminate lyase, putative
BLJ_0021	26.83	VFG0307	Neutrophil activating protein (bacterioferritin)
BLJ_1889	24.14	VFG2227	O-antigen export system permease protein
BLJ_1251	26.05	VFG1461	ORF A protein
BLJ_0214	30.5	VFG0594	Pathogenicity island encoded protein: SPI3
BLJ_0159	33.25	VFG0594	Pathogenicity island encoded protein: SPI3
BLJ_1474	57.32	VFG1386	phoP
BLJ_1703	25.65	VFG2220	Phosphoglucomutase
BLJ_0497	28.35	VFG2362	Phosphomannomutase
BLJ_1137	25.1	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_0508	25.93	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_1453	29.27	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_0408	38.22	VFG2059	ATP-binding component of ABC transporter
BLJ_0480	27.04	VFG2061	Phosphoprotein phosphatase
BLJ_0805	28.09	VFG1384	proC
BLJ_1396	31.06	VFG1384	proC
BLJ_0584	26.09	VFG1387	purC
BLJ_1772	22.28	VFG0480	Putative amino acid permease
BLJ_0538	25.17	VFG0480	Putative amino acid permease
BLJ_1329	24.42	VFG1965	Putative aminotransferase
BLJ_0025	30.45	VFG2301	Putative carbonic anhydrase
BLJ_0922	23.51	VFG0031	Putative glycosyl transferase
BLJ_1670	38.88	VFG1668	Putative lysil-tRNA synthetase LysU
BLJ_0563	25	VFG1498	Putative periplasmic solute binding protein
BLJ_1171	28.48	VFG0483	Putative regulatory protein, deoR family
BLJ_1517	29.25	VFG0483	Putative regulatory protein, deoR family
BLJ_0344	37.02	VFG1702	Putative response regulator
BLJ_0040	27.91	VFG1746	Putative two-component response regulator
BLJ_0740	29.13	VFG1746	Putative two-component response regulator
BLJ_1105	24.49	VFG0168	Pyochelin biosynthesis protein PchD
BLJ_0409	25.56	VFG0168	Pyochelin biosynthesis protein PchD
BLJ_0720	41.04	VFG0479	Pyruvate kinase I (formerly F), fructose stimulated
BLJ_1163	55.32	VFG1826	relA
BLJ_0995	25.84	VFG1889	Response regulator GacA
BLJ_1679	28.89	VFG1889	Response regulator GacA
BLJ_1083	40.89	VFG0473	Response regulator in two-component regulatory system with BasS
BLJ_1273	26.57	VFG1115	ROK family protein
BLJ_1620	26.62	VFG1115	ROK family protein
BLJ_1622	31.35	VFG1115	ROK family protein
BLJ_1796	27.31	VFG0526	Salmonella iron transporter: fur regulated
BLJ_0662	29.06	VFG0526	Salmonella iron transporter: fur regulated

BLJ_0712	25.4	VFG0528	Salmonella iron transporter: fur regulated
BLJ_1174	51.39	VFG1405	sigA
BLJ_1258	41.15	VFG1412	sigH
BLJ_1342	33.11	VFG2161	Signal peptidase Ⅱ
BLJ_0906	21.73	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1923	22.38	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1360	22.88	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1421	23.24	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0611	23.31	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1836	23.32	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0459	23.33	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1278	23.43	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1998	23.51	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1522	23.6	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0109	23.63	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0418	23.69	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0118	23.7	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0520	23.85	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1976	24.27	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0099	24.31	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1605	24.34	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0132	24.53	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1912	24.58	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0912	24.69	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0318	24.71	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1515	24.93	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1933	25	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1718	25	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1997	25.36	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0400	25.37	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0515	27.08	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1321	28.21	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1232	29.83	VFG1028	Tn21 integrase IntI1
BLJ_1160	43.1	VFG2168	Transcriptional regulator, Cro/CI family
BLJ_0747	28.98	VFG1122	Transposase ORFAB, subunit B
BLJ_1180	43.64	VFG1398	trpD
BLJ_1871	39.62	VFG1967	UDP-galactopyranose mutase
BLJ_1644	39	VFG2361	UDP-glucose 4-epimerase
BLJ_1680	54.49	VFG2361	UDP-glucose 4-epimerase
BLJ_1891	52.63	VFG0963	UDP-glucose 6-dehydrogenase
BLJ_0697	46.15	VFG1414	whiB3

MIC: Minimum inhibitory concentration; ABC: ATP-binding cassette.

Table 4 Putative genes associated with adhesion identified in the genome of *Bifidobacterium longum* JDM301

Locus_tag	Pfam number	Product name
BLJ_1932	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0112	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1284	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1420	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0131	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1604	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1686	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1964	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1994	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1996	pfam01547	Family 1 extracellular solute-binding protein
BLJ_2001	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0288	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0321	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0345	pfam01547	phosphate ABC transporter periplasmic phosphate-binding protein
BLJ_0414	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0522	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0523	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0524	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0012	pfam07174	Hypothetical protein BLJ_0012
BLJ_1801	pfam05738	LPXTG-motif protein cell wall anchor domain-containing protein
BLJ_0140	pfam07811	TadE family protein

with iron, amino acid and sugar transport, 5 transposases, and 2 glutamine synthetase related to plasminogen (Plg)-binding (Table 3).

Although the ability to adhere to the intestinal wall has been one of the selection criteria for probiotics and also a characteristic of commensal bacteria in the intestine, adhesion is also considered to be a significant step in the initial pathogen infections[26]. Thus, predicted proteins for adhesion of JDM301 were also included in the analysis of virulence. A total of 21 predicted proteins for adhesion were identified in JDM301 (Table 4). A large number of predicted surface and extracellular proteins were identified in JDM301, which may be involved in the bacterium-host interaction as in other LAB[27]. A total of 217 proteins with probable Sec-type signal peptides were identified by the tool, Signal P[28]. The genome of JDM301 also harbors 18 copies of extracellular solute-binding protein (SBP, pfam01547) which is predicted to bind oligosaccharides (SBP family 1) as a component of the ABC transporter complex.

DISCUSSION

As more probiotic strains are used in the food and drug industry, more attentions should be paid to the safety of strains used as probiotics. Thus, the safety of LAB used as probiotics need to be reassessed using the latest technology. *B. longum* JDM301, is a commercial probiotic strain used in many probiotic products sold in China. Analysis of the genome of JDM301 reveals several potential risk factors needing further experimental validation, including a tetracycline resistance gene (*tetW*) with the risk of transfer, and the genes associated with harmful metabolites.

Bifidobacteria were considered free of phage infection until prophage-like elements were identified in the genomes of *B. longum* NCC2705, *B. longum* DJO10A and *B. breve* UCC2003[29]. Absence of complete prophages is important for the stability of genomes and for industrial applications of probiotic bacteria[21,30]. Absence of complete prophages and scarcity of IS element may play important roles in promoting genome stability of JDM301[31]. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system (CASS), which is involved in defense against phages and plasmids[32]. No CRISPR was discovered in the genome. R-M systems are diverse and widespread in nature and they are considered as barriers to HGT, e.g., in transformation and phage infection[33]. The diversity of R-M systems in *B. longum* JDM301 may be significant to the stability of genome and its use in industry compared with the other two *B. longum* strains.

B. longum JDM301 was not resistant to tetracycline as the minimum inhibitory concentration (8.0 mg/L) was not higher than the breakpoint value (8.0 mg/L)[34]. However, the MIC for *B. longum* strains ranges from 0.5 to 2 mg/L in a report[35]. Thus, further experiments may be needed to determine the microbiological breakpoint. The *tetW* (BLJ_1245) gene encodes for a ribosomal protection protein and *tetW* genes were responsible for acquired tetracycline resistance in human *B. longum* strains[36]. The rest of the tetracycline resistance genes found in *B. longum* JDM301 were *tetV* (BLJ_0814), *tetQ* (BLJ_1401) and *tetPB* (BLJ_0594). The gene *tetV* encodes for a tetracycline efflux pump and the genes *tetQ* and *tetPB* encode for ribosomal protection proteins. Further experiments are needed to confirm whether the *tetW* gene in the chromosome of *B. longum* JDM301 is a transferable antibiotic resistance determinant and responsible for resistance to tetracycline in human *B. longum* strains.

The MIC of *B. longum* JDM301 to bacitracin was 26.7 mg/L, which indicated a moderate resistance. A previous report[25] indicated that *B. longum* strains were susceptible to bacitracin. A total of 7 putative bacitracin resistance genes were identified, including 6 genes encoding for ABC transporters and 1 for an uncharacterized bacitracin resistance protein. These genes may be responsible for the resistance to bacitracin.

The resistances to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptibility of JDM301 to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol were consistent with reported findings[22-25,36]. However, there are discrepancies between the phenotype and the genotype. *B. longum* JDM301 was sensitive to vancomycin and chloramphenicol but the genome contained vancomycin and chloramphenicol resistance genes. Further analysis will be needed to determine this discrepancy.

Several cases of D-lactic acidosis associated with consumption of LAB in patients with short bowel syndrome were reported[37,38], implying that bacteria used as probiotics should be screened for the ability to generate D-lactate. In this study, two homologs of DLD genes were identified in the genome of JDM301. Since there were no reported cases of D-lactic acidosis caused by bifidobacteria[37-39], the activities of these homologous DLDs in bifidobacteria may be low so that the amount of lactate produced is insufficient to cause D-lactic acidosis.

Although biogenic amines (BA) play an important physiological role in mammals, a high amount of BA in the diet may have a variety of toxic effects[40]. The main BA contained in food and beverage includes histamine, tyramine, putrescine, and cadaverine, some of which are associated with toxicological characteristics of food poisoning[41]. The decarboxylase activities of histidine, tyrosine and ornithine were reported in lactobacilli and the capabilities might be strain-dependent rather than species-dependent[42]. Therefore, BA production, especially thylamine and tyramine, must be carefully evaluated for individual strains.

Bacterial enzymes, such as GN, GS, NR, AR and AS, play important roles in the metabolism of carcinogens and other toxicants in the intestine. Homologs of GS are common in sequenced *Bifidobacteria* genomes where GS and GN facilitate the absorption of a variety of toxicants and may contribute to the development of colon cancer. The link between *Bifidobacteria* and the genotoxic enzyme activities of intestinal microflora has been reported[43,44], with *Bifidobacteria* inhibiting the activity of some genotoxic enzymes[45]. NR activity is common in oral bacteria and it plays an important role in bacterial nitrate reduction. Although NR activities have been reported in *Bifidobacteria*, the activity of this enzyme is lower than the NR activity of other gut bacteria[6].

CBSH mediates microbial bile tolerance and enhances microbial survival in the intestine[46]. Metagenomic analyses demonstrated that CBSH activity is enriched in the human gut microbiome, and has the potential to greatly influence host physiology[46]. In *Bifidobacterium* spp. and *Lactobacillus* spp., CBSH activity is also common and nearly all *Bifidobacteria* species and strains have bile salt hydrolase activities[47]. However, bile salt hydrolase activity releases free bile acids which are harmful to the human body and may act as mutagens[48,49]. Recommendations have been made for absence of bile salt transformation capacity in bacteria added to food[50]. However, it is noteworthy that the evidence for harmful effects is inconclusive so far and bile salt deconjugation activity may play a role in reducing human serum cholesterol[51]. Given the huge CBSH pool in intestinal microflora, the CBSH activities of the small number of additional bacteria consumed as probiotics can be ignored[48].

Putative genes for Plg-binding proteins, DnaK (BLJ_0123) and glutamine synthetase (BLJ_0624 and BLJ_1324) were found in the JDM301 genome, where these proteins play a role in the interaction with human epithelial cells. The protein DnaK has been shown to be present on the surface of pathogens, such as *Neisseria meningitides*[52]. The glutamine synthetases BLJ_0624 and BLJ_1324 had a 62.11% and 32.49% similarity to the glutamine synthetases in *Mycobacterium tuberculosis* H37 Rv. In

the presence of Plg activators, Plg binding to the bacterial surface is converted to plasmin, which is a broad-spectrum serine protease involved in degradation of fibrin and noncollagenous proteins of extracellular matrices and activates latent procollagenases[53]. It is believed that the capability to intervene with the Plg/plasmin system of a host is a strategy for host colonization and bacterial metastasis shared by several pathogens and commensals of the human intestinal tract[53,54]. The plasminogen-dependent proteolytic activity of *B. lactis* BI07 and *B. longum* was shown to be dose-dependent[55,56].

A homolog (BLJ_0880, 24.18% identity) of a gene encoding a component in ferric dicitrate uptake system (Fec) of *Shigella flexneri* serotype 2a, FecE, was identified in the genome of JDM301. As an iron uptake system, Fec is critical for bacterial survival and plays an important role in bacterial virulence[57]. In addition, BLJ_1105 and BLJ_0409 proteins associated with iron acquisition in JDM301 were 24.49% and 25.56% similar to pyochelin biosynthesis protein in *Pseudomonas aeruginosa,* and BLJ_0712, BLJ_1796 and BLJ_0662 proteins were 25.4%, 27.31 and 29.06% similar to iron transporters of *Salmonella enterica*.

The human pathogen, *Helicobacter pylori*, produces a neutrophil activating protein (NAP) which activate human leukocytes and induces an inflammation, which facilitates the growth of the pathogen[58]. A homolog (BLJ_0021; 26.83% identity) of the gene encoding a NAP was identified in the genome of JDM301.

In JDM301, BLJ_0012 encodes a protein harboring fibronectin-binding motif (Pfam number 07174) that allows mycobacteria to bind to fibronectin in the extracellular matrix and may mediate the adhesion of JDM301 to its host[59]. A potential protein for *Bifidobacteria* adhesion to intestinal cells is the putative LPXTG-motif protein with collagen binding motifs (Cna_B, pfam05738) encoded by BLJ_1801, which shows a 34% identity to a predicted fimbrial subunit in the genome of *B. dentium* Bd1. This protein may be involved in the recognition of and adhesion to mucosal epithelial cell surfaces[19]. Its homologous proteins were also identified in the genome sequences of both *B. longum* NCC2705 and *B. longum* DJO10A genomes[3,60]. *B. longum* subsp. *infantis* 15697, *B. longum* NCC2705 and *B. adolescentis* contains 21, 10 and 11 copies of extracellular solute-binding protein, respectively[3,4]. Comparably, the SBP family 1 proteins are more abundant in JDM301 than the three other *Bifidobacteria* strains due to the genome size.

Finally, JDM301 encodes a number of proteases and peptidase that may contribute to virulence owing their ability to degrade host proteins for bacterial nutrition sources[61]. However, not all the genes associated with virulence have been known until now. Thus, despite the evaluation based on the whole genome sequences, it is recommended that the rat endocarditis and the immunocompromised mouse model should be used for *in vivo* assessment of safety for the low pathogenicity of LAB[48].

Recently, there has been more interest in using probiotic products to promote health and treat diseases. Probiotics have been investigated in clinical trials, such as treatment for diarrhea, D-lactic acidosis, necrotizing enterocolitis, inflammatory bowel disease and so on[39,62-64]. The mechanisms by which probiotics exert their effects are still obscure, which may include modification of gut pH, antagonism of pathogens, modulation of immunity as well as supplements of some nutrients[65]. However, safety issues of probiotics have been discussed in many reports[5,48]. There are reported cases of infections associated with probiotic strains[5]. Although the strain is safe based on phenotype, the information derived from complete bacterial genome sequences reveals some putative unfavorable genes, such as genes encoding for Plg-binding proteins, proteases and genes associated with production of D-lactate. In addition, patients are generally more susceptive to infection and harmful metabolites, such as D-lactate than healthy persons. Thus, the biosafety of probiotics, especially strains used in therapy, must be assessed more carefully and comprehensively.

In conclusion, this study compared the genome of JDM301 with other *Bifidobacteria* and assessed the genomic stability, the potential for antibiotic resistance, the potential for virulence and the potential production of harmful metabolites of this strain. The core genome of *B. longum* is composed of 1265 genes, and 219 genes are unique in JDM301. Our data showed putative virulence genes in the genomes of JDM301 as well as putative genes associated with production of harmful metabolites. In addition, a potentially transferable antibiotic resistance gene was detected in the chromosome of JDM301, which needs to be experimentally validated. This assessment provides information on potential risk factors, which should be further evaluated experimentally, e.g., *in vivo* assessment using animal models.

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COMMENTS

Background

Bifidobacterium longum JDM301 is a commercial strain used widely in China with several probiotic functions. Recently, there has been more interest in using probiotic products to promote health and treat diseases. As model probiotic bacteria, *Bifidobacteria* are often added to probiotic products in combination with other lactic acid bacteria. The biosafety of probiotic bacteria is attracting more attentions with its enlarged applications. As more commercial probiotic products are being introduced in the market, it is necessary to reassess the safety of these probiotic products using the latest technology.

Research frontiers

With a long and safe history of application, lactic acid bacteria have acquired the status of "Generally Regarded As Safe". However, published reports of rare infections involving *Lactobacilli* or *Bifidobacteria* are available. The strains selected as probiotics are needed to be assessed carefully and comprehensively. This study may contribute to a better biosafety assessment of probiotic bacteria.

Innovations and breakthroughs

This is the first study to assess the biosafety of probiotic bacteria based on

complete genome sequences. Through bioinformatics analysis of the genome sequences, the authors found that although the strain was safe based on phenotype, the information derived from complete bacterial genome sequences revealed some putative unfavourable genes that should be paid attention to.

Applications

The study provides a comprehensive assessment on potential risk factors of a probiotic strain based on complete genome sequences. The information related to biosafety derived from the genome of JDM301 will contribute to a wider and deeper insight into the safety of probiotic bacteria.

Peer review

This is a very nice and comprehensive study assessing the genomic stability, potential of antibiotic resistance, virulence and production of harmful metabolites. This adds valuable information to current knowledge about probiotics.

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Potential implications of *Helicobacter pylori*-related neutrophil-activating protein

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Abstract

Helicobacter pylori (*H. pylori*) virulence factors promote the release of various chemoattractants/inflammatory mediators, including mainly the neutrophil-attractant chemokine interleukin-8 and neutrophil-activating protein (NAP), involved in *H. pylori*-induced gastric pathologies. Co-administration of Chios mastic gum (CMG), which inhibits *H. pylori* NAP, with an *H. pylori* eradication regimen might add clinical benefits against *H. pylori*-related gastric pathologies, but possibly not CMG as main therapy. Although *H. pylori* NAP and other *H. pylori*-related cytotoxins [i.e., vaculating cytotoxin (VacA)] appear to play a major role in generating and maintaining the *H. pylori*-associated gastric inflammatory response and *H. pylori* NAP is a promising vaccine candidate against *H. pylori* infection (*H. pylori*-I), concerns regarding its potential drawbacks, particularly neurogenic ones, due to possible cross-mimicry, should be considered. Possible cross-mimicry between *H. pylori* NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica patients. Moreover, the sequence homology found between *H. pylori* VacA and human Na+/K+-ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients. A series of factors have been implicated in inducing blood-brain barrier (BBB) disruption, including inflammatory mediators (e.g., cytokines and chemokines induced by *H. pylori*-I) and oxidative stress. BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, thereby playing a major role in multiple sclerosis pathogenesis. Relative studies show a strong association between *H. pylori*-I and MS. *H. pylori*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and perpetuating neural tissue damage. Finally, *H. pylori* NAP also plays a possible pathogenetic role in both gastric and colon oncogenesis.

Key words: *Helicobacter pylori*; Neutrophil-activating protein; Chios mastic gum; Cross-mimicry; Multiple sclerosis; Demyelination; Gastric carcinogenesis

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TO THE EDITOR

In their recent paper published in this journal, Choli-Papadopoulou *et al*[1] consider the development of new drugs targeting *Helicobacter pylori* (*H. pylori*) neutrophil-activating protein (NAP) and this raises some concerns.

With reference to a study[2] focusing on *H. pylori* NAP-mediated neutrophil activation before and 2 mo after *per os* administration of Chios mastic gum (CMG), the authors claimed that "these results indicate a substantial down-regulation of the innate cellular immune effectors, which, according to unpublished clinical data in the context of this study, are accompanied by a significant clinical improvement of the patients' complaints (dyspepsia, epigastric discomfort, distention)"[1]. However, such clinical benefits cannot be deduced from this study[2] and, as mentioned, relative clinical data on CMG as treatment for *H. pylori* and peptic ulcer are controversial[2]. Although *H. pylori* virulence factors promote the release of various chemoattractants/inflammatory mediators including mainly the neutrophil-attractant chemokine interleukin-8 and *H. pylori* NAP involved in *H. pylori*-induced gastric pathologies[3], our clinical experience suggests that only co-administration of CMG with an *H. pylori* eradication regimen might add clinical benefits against *H. pylori*-related gastric pathologies, but possibly not CMG as main therapy, as the authors claimed[1,2]. In particular, co-administration of CMG might be a potential therapy to reduce damage of gastric mucosa induced by *H. pylori* NAP. However, large-scale relative prospective studies are needed to elucidate this field.

The authors, further considering data on the safety and immunogenicity of a vaccine comprising *H. pylori*-induced vaculating cytotoxin (VacA), cytotoxin associated gene and *H. pylori* NAP, suggested that the obtained neutrophil activation by the C-terminal region of *H. pylori* NAP opens new pathways for drug design directed at *H. pylori* inflammation[1]. In particular, both VacA and *H. pylori* NAP play a major role in generating and maintaining the *H. pylori*-associated gastric inflammatory response, and *H. pylori* NAP is a promising vaccine candidate against *H. pylori* infection (*H. pylori*-I). However, concerns regarding potential drawbacks of *H. pylori* NAP, particularly neurogenic ones, should be considered. For instance, possible cross-mimicry between *H. pylori* NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica (NMO) patients. In this regard, by using histology, the practical gold standard for the diagnosis of *H. pylori*-I, we have shown a strong association between *H. pylori*-I and MS[4]. *H. pylori*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and perpetuating neural tissue damage[4]. In this respect, *H. pylori* NAP, as a virulence factor, recruits leukocytes from the vascular lumen, and activates neutrophils, monocytes and mast cells, as mentioned by the authors. Besides, the sequence homology found between *H. pylori* VacA and human Na+/K+-ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients[5]. Moreover, VacA exhibits chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces BMDMCs to produce pro-inflammatory cytokines[5]. A series of factors have been implicated in inducing blood-brain barrier (BBB) disruption, including the aforementioned inflammatory mediators (e.g., cytokines and chemokines induced by *H. pylori*-I) and oxidative stress. BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, thereby playing a major role in MS/NMO pathogenesis[6]. Therefore, *H. pylori* NAP and *HP*-I itself, by inducing several mediators, may influence MS/NMO (including relapsing type) pathophysiology, thereby raising possible concerns regarding even the C-terminal region of *H. pylori* NAP use as a candidate vaccine. Accordingly, relative studies are also needed to clarify the aforementioned concerns.

Finally, the possible *H. pylori* NAP pathogenetic role in gastric carcinogenesis, mentioned by the authors, may also apply to colon oncogenesis[2,7].

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May 3-5, 2012
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Amman, Jordan

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May 17-21, 2012
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May 18-19, 2012
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San Diego, CA 92101, United States

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Pancreatic Cancer: Progress and Challenges
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July 25-26, 2012
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September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
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Florida Gastroenterologic Society 2012 Annual Meeting
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Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
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November 9-13, 2012
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

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.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

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

Issue with no volume

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

No volume or issue

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

Books

Personal author(s)

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

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

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

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

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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