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[2]Passed away on June 11, 2007
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Anal transition zone in the surgical management of ulcerative colitis

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Abstract

Preservation of the anal transition zone has long been a significant source of controversy in the surgical management of ulcerative colitis. The two techniques for restorative proctocolectomy and ileal pouch anal anastomosis (RPC IPAA) in common practice are a stapled anastomosis and a handsewn anastomosis; these techniques differ in the amount of remaining rectal mucosa and therefore the presence of the anal transition zone following surgery. Each technique has advantages and disadvantages in long-term functional outcomes, operative and postoperative complications, and risk of neoplasia. Therefore, we propose a selective approach to performing a stapled RPC IPAA based on the presence of dysplasia in the preoperative endoscopic evaluation.

Key words: Anal transition zone; Ileal pouch anal anastomosis; Restorative proctocolectomy; Ulcerative colitis

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INTRODUCTION

Restorative proctocolectomy and ileal pouch anal anastomosis (RPC IPAA) remain the standard of care in the surgical management of ulcerative colitis; however, controversy persists regarding preservation of the anal transition zone (ATZ). RPC IPAA is performed *via* either a stapled technique or a transanal mucosectomy and endoanal anastomosis, frequently referred to as a handsewn anastomosis. When the stapled technique is utilized, the ATZ is preserved; whereas a mucosectomy is usually performed from the level of the dentate line, therefore eliminating the ATZ and the proximal cuff of rectal epithelium. While the preservation of the ATZ has been shown to improve functional results and reduce operative time and complications, great debate persists regarding outcomes during recurrent or persistent disease and the theoretical risk of malignant transformation. Given the paucity of data in this realm, the long-term fate of the ATZ in the surgical management of ulcerative colitis has yet to be determined.

DEFINING THE ATZ

Defining the anatomy of the ATZ is difficult. Fenger first described the ATZ as "the zone interposed between uninterrupted crypt bearing colorectal-type mucosa above and uninterrupted squamous epithelium below"[1], which he characterized utilizing an Alcian dye technique. This technique is used for staining the ATZ macroscopically as the columnar epithelium stains dark blue, the squamous epithelium does not stain, and the ATZ stains pale blue. The Alcian dye technique delineates the margins of the ATZ from 6 mm below to 20 mm above the dentate line[1] with the median span from 0.73 to 0.89 cm[1,2]. Further studies by Thompson-Fawcett demonstrated that the Alcian dye technique overestimates the length of the ATZ when comparing this to computer mapping of the histological results[2]. Using computer histological mapping, the median upper and lower borders of the ATZ, measured from the

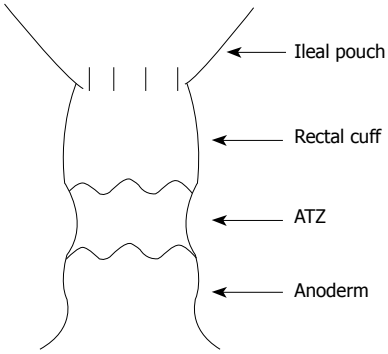

Figure 1 Retained rectal cuff after RPC IPAA.

lower margin of the internal sphincter, were 1.82 and 1.27 cm, respectively, with the dentate line measuring 1.05 cm by histology or 1.16 cm macroscopically above the lower margin of the internal sphincter. The median span was 0.45 cm *versus* 0.73 cm by the Alcian dye technique. This difference in length was accounted for by close examination of the histological specimens. The pale blue zone was due to staining of both superficial nuclei of thin squamous anoderm and the transitional epithelium that characterizes the ATZ. Fenger's technique for analyzing the ATZ did not exactly match the macroscopic Alcian blue specimens to the histological specimens, in comparison to Thompson-Fawcett's computer histological and Alcian dye mapping, and therefore likely overestimated the ATZ.

After careful measurements aided by computer mapping, the ATZ is now recognized to be smaller than previously considered. Significantly, columnar epithelium exists lower in the anorectal canal, and it is left behind when a stapled technique is utilized, which could be of consequence following RPC IPAA (Figure 1).

PRESERVATION OF THE ATZ: FUNCTIONAL OUTCOMES AND COMPLICATIONS

Duthie and Gairns in 1960 described a vast array of sensory nerve endings in the anal canal[3], which demonstrated sensitivity to temperature, touch, and pain, while the rectal mucosa lacked this innervation and sensation[3,4]. The rectum is able to sense distension, however, that results in a brief reflexive relaxation of the internal anal sphincter and contraction of the external anal sphincter, thus allowing the anal mucosa to sample the rectal contents. This sampling is thought to aid the ATZ in discrimination between gas, liquid, and solid stool. Anorectal sensation is abnormal in incontinent patients[5,6] and in patients following mucosectomy[7,8]. Thus, the extensive innervation of the ATZ is thought to play a very important role in maintaining fecal continence.

Following RPC IPAA *via* stapled or mucosectomy with endoanal anastomosis techniques, patients demonstrate decreased anal resting pressure (ARP)[9-13], thought to occur secondary to the extent of dissection, level of rectal transection, diameter of the stapling device, and anal retraction. However, ARP is greater following RPC IPAA stapled anastomoses than following mucosectomy with endoanal anastomoses[8,10,11,14,15]. Controversy exists regarding improvement of ARP over time when comparing the two surgical techniques. ARP has been demonstrated to return to a normal value by 12 mo after the operation only in RPC IPAA *via* a stapled technique[9], whereas Tuckson *et al* demonstrated no significant improvement in ARP 13 mo after either technique[10]. Both these studies analyzed a very small group of patients, with relatively short follow-up.

Functionally, preservation of the ATZ *via* RPC IPAA stapled anastomosis improves clinically relevant outcomes. Three prospective randomized controlled trials were performed in the early 1990s with limited numbers of patients (between 23 and 41 in each study). The results showed only limited benefit for stapled anastomosis with no worse outcomes[15-17]. More specifically, Luukkonen *et al* demonstrated no statistically significant differences between the only two functional parameters measured: number of nocturnal bowel movements and frequency of mucous leakage[17]. Choen *et al* also argued that many functional parameters showed no differences including frequency of bowel movements, number of nocturnal bowel movements, ability to delay defecation, and full continence[16]. However, Reilly *et al* demonstrated trends towards improved functional outcomes in all parameters measured: nocturnal seepage, daytime continence, ability to differentiate stool and flatus, and pad usage[15].

Other studies more clearly outline the functional benefit of stapled anastomoses. Remzi *et al* showed that patients with stapled anastomoses had better outcomes in every functional category measured, including statistically significant differences in incontinence, daytime and nighttime seepage, and pad usage[18]. Saigusa *et al* described an improved ability to discriminate between flatus and stool in the stapled *versus* handsewn group, 80% *versus* 33% ($P < 0.05$)[8]. Michelassi *et al* found that between 57% and 78% of patients were always able to delay a bowel movement until convenient; although this did not differ between the two groups[19]. These authors also demonstrated that stapled patients had improved rates of full continence, which persisted over time. Sagar *et al* studied stapled IPAA patients only for 12 mo postoperatively and compared parameters at 3 and 12 mo[9]. They showed that not only were functional outcomes better in the stapled IPAA group, but outcomes improved over time. Statistically significant improvements in the number of bowel movements ($P < 0.001$), number of nocturnal bowel movements ($P < 0.001$), frequency of loose stool consistency ($P < 0.001$), use of anti-diarrheal medication ($P < 0.01$), ability to defer defecation for more than 15 min ($P < 0.001$) and ability to discriminate flatus from stool ($P < 0.001$) were demonstrated. A single meta-analysis of 4183 patients (2699 handsewn and 1484 stapled IPAA) demonstrated no significant differences in the number of bowel movements, number of nocturnal bowel movements, daytime seepage, and use of anti-diarrheal

medication[14]. However, incontinence was more common in the handsewn group ($P = 0.009$) and the incidence of nocturnal seepage and nocturnal pad usage favored the stapled IPAA group ($P < 0.001$ and $P = 0.007$, respectively). Both groups were rated with a high quality of life with no statistically significant difference between the groups.

Early septic complications can occur following stapled or handsewn IPAA and may include anastomotic dehiscence with diffuse or localized sepsis, abscess, and fistula formation. These septic complications may require prolonged hospitalization, interventional procedures, reoperation, and pouch excision, therefore resulting in increased patient morbidity, prolonged recovery, and loss of bowel continuity. Risk factors for pelvic sepsis include ulcerative colitis, ulcerative colitis associated with toxic megacolon or fulminant colitis, male gender, and a handsewn anastomosis[20]. More than a two-fold increase in the rate of septic complications has been reported following handsewn *versus* stapled anastomoses[14,20,21], which results in increased rates of pouch failure requiring permanent diverting ileostomy[14,20,22] and more frequent pouch excision[14,21]. Pouch failure and pouch excision occur secondary to anastomotic dehiscence, poor functional results, pouchitis, perianal disease, and pouch leakage. Risk factors for pouch failure have been outlined and include handsewn anastomoses, anastomotic tension, use of diverting ileostomy, Crohn's disease, and postoperative anastomotic leak[22]. Further supporting the use of a stapled technique, when anastomotic leaks do occur, a better prognosis has been shown following a stapled anastomosis[20,22].

INFLAMMATION AND DYSPLASIA IN THE ATZ

It is well documented that chronic inflammation may lead to dysplasia and dysplasia may ultimately lead to neoplasia in patients with long-standing ulcerative colitis. The retained ATZ following stapled RPC IPAA is therefore at risk for chronic inflammation from recurrent or persistent disease, dysplasia, and possibly malignancy. The retained ATZ and potentially rectal mucosa is greater in length in the stapled anastomosis patient but can still be present in the handsewn patient due to the variation in location of the ATZ and incomplete transanal mucosectomy[2,23,24].

Inflammation within the ATZ is well documented. In early studies the incidence of endoscopic anal canal inflammation confirmed by biopsy was reported to be as high as 22% as estimated by Lavery *et al*, while the incidence of symptomatic inflammatory changes in the retained mucosa was 14.7%[25]. More recent prospective data document the incidence of inflammation following stapled RPC IPAA to be much higher: 4.6% with acute inflammation, 84.9% with chronic inflammation, and only 10.5% with normal mucosa[26]. The presence of inflammation, however, does not seem to negatively affect functional outcomes, as our group has reported in a large prospective study of 225 patients, with 96% of them reporting perfect fecal continence, 5.3% using protective pads, and 93.2% being able to delay a bowel movement for more than 30 min[26]. Patients with chronic inflammation of the ATZ still report better functional parameters when compared to handsewn control patients, including statistically significant improved flatus *versus* stool discrimination, decreased protective pad usage, and decreased dietary modification regarding meal timing[27]. In addition, patients with chronic inflammation of the retained ATZ more commonly reported an improved quality of life than prior to surgery when compared to handsewn patients ($P < 0.001$)[27].

Several studies have published their experience with postoperative serial endoscopic surveillance and the risk of dysplasia following preservation of the ATZ *via* a stapled RPC IPAA. The principal risk factor for developing dysplasia in the ATZ was the presence of dysplasia or cancer in the proctocolectomy specimen, independent of the location; no other factors including age, sex, or duration of disease, appear to increase this risk[28-31]. Ziv *et al* reported eight cases of dysplasia in 254 patients with a mean postoperative follow-up of 2.3 years; however, repeat biopsy revealed dysplasia in only two of these patients, who underwent a transanal mucosectomy without evidence of cancer in the final specimen[28]. Remzi *et al* analyzed a total of 178 patients with a mean follow-up of more than 10 years and identified dysplasia in only eight patients (4.5%) and no evidence of cancer[30]. No dysplasia was identified by our group in 242 patients with a mean follow-up of 56 mo[27]. This small disparity in incidence may be explained by variation in patient selection. In our practice, we do not offer a stapled RPC IPAA to patients with documented, and confirmed by two independent pathologists, colorectal dysplasia, irrespective of degree and location. Instead, these patients will undergo a transanal mucosectomy with a handsewn anastomosis[27]. We believe that the presence of dysplasia irrespective of degree and location is an indication of "mucosal instability." Since the primary risk factor for developing dysplasia in the ATZ is the presence of dysplasia or cancer in the surgical specimen, we perform a complete mucosectomy in this patient population. Complete absence of dysplasia or cancer in the ATZ in our series after 56 mo of follow-up, supports this approach.

Overall, dysplasia within the ATZ is uncommon and the risk of developing cancer following RPC IPAA is even more unlikely with only 19 reported cases in the literature[31] (Table 1). Even following a mucosectomy with endoanal anastomosis, islands of rectal mucosa probably exist at or below the dentate line in at least 20% of patients[23,24], These islands of rectal tissue may not be easily visualized during endoscopic biopsy, and dysplastic or malignant transformation may ultimately be difficult to detect. In fact, of the 19 reported cases of adenocarcinoma, 13 occurred in patients who underwent transanal mucosectomy. Furthermore, six of these patients developed cancer within the pouch itself, raising concern for metaplasia of the pouch as a method for

Table 1 Adenocarcinoma after RPC IPAA for UC[1]

Reference	Yr	RPC IPAA	Preoperative diagnosis	Pathological diagnosis	Yr to CA[2]	Site of carcinoma
Ravitch	1984	HS	NR	NR	NR	NR
Stern	1990	HS	Dysplasia	HGD rectum	3	Pouch
Puthu	1992	NR	NR	NR	6	NR
Rodriguez-Sanjuan	1995	HS	Dysplasia	HGD rectum	3.5	Pouch
Sequens	1997	Stapled	Carcinoma	CA rectum	1	ATZ
Vieth	1998	HS	Carcinoma	CA colon, multifocal dysplasia	2	Pouch
Iwama	2000	HS	UC	LGD	18	Anastomosis
Rotholtz	2001	Stapled	UC	HGD distal margin	7	ATZ
Heuschen	2001	HS	Dysplasia	CA colon	3	Pouch
Laureti	2002	HS	Carcinoma	CA anastomosis	2	Anastomosis
Hyman	2002	Stapled	Dysplasia	HGD distal margin & colon	5	Rectal stump
Baratsis	2002	Stapled	UC	CA cecum, multifocal dysplasia	2	ATZ
Bentrem	2003	HS	UC	CA colon, dysplasia	14	Pouch
Hassan	2003	HS	UC	UC	2	Pouch
Negi	2003	HS	Dysplasia	HGD	5	Rectal stump
Bell	2003	Stapled	Dysplasia	HGD colon	12	Anastomosis
Lee	2005	HS	Dysplasia	HGD rectum	2	Anastomosis
Lee	2005	HS	Dysplasia	CA rectum	6.5	Anastomosis
Lee	2005	HS	UC	UC	16	Rectal stump
Schaffzin	2005	HS	NR	NR	25	Pouch
Knupper	2006	Stapled	UC	NR	3	Pouch
Walker	2006	HS	NR	Dysplastic colon & rectum	17	Pouch
Das	2007	HS	UC	UC	25	ATZ
Ota	2007	HS	NR	UC	7	Rectal stump
Ruffolo	2007	Stapled	Carcinoma	CA colon at two sites	3	ATZ
Koh	2008	Stapled	NR	NR	14	Pouch inlet
Pedersen	2008	HS	HGD	CA colon, dysplastic colon	11	Rectal stump
Chia	2008	Stapled	UC	CA	3	ATZ

[1]Excludes articles not in English and one study using Cavitron Ultrasonic Surgical Aspirator technique for rectal mucosal stripping; [2]Yr to presentation of carcinoma after RPC IPAA; HS: Handsewn anastomosis; Stapled: Stapled anastomosis; NR: Not recorded or unknown; UC: Ulcerative colitis, HGD: High grade dysplasia; CA: Carcinoma; LGD: Low grade dysplasia.

malignant transformation. Yet, Swedish research has demonstrated that although the pouch itself may undergo metaplasia, there has been no progression to carcinoma; dysplasia occurred in less than 4.4% of patients; no high-grade was observed; and experienced pathologists did not even agree on the presence of dysplasia itself[32]. None of these patients had documented dysplasia or carcinoma in the surgical specimen preoperatively. When reviewing the cases of carcinoma following RPC IPAA, it is important to note that all but three patients had documented dysplasia or carcinoma within the original surgical specimen[31]. Given these data and the extensive evidence for the development of carcinoma in ulcerative colitis patients following dysplastic changes of the colon, we believe that it is appropriate to label dysplasia as a marker for "mucosal instability." Therefore, it is our belief that the presence of dysplasia or carcinoma, irrespective of the severity or location, should preclude a stapled RPC IPAA.

The presence of dysplasia or malignancy within the ATZ raises concern regarding treatment. Invasive malignancy requires a complete IPAA excision and end ileostomy. On the other hand, clear data regarding management of dysplastic changes within the ATZ do not exist. Dysplasia may even sometimes be self-limiting. Interestingly, several studies have shown regression of low-grade and even high-grade dysplasia to normal mucosa in some patients by serial biopsies[28,30], which is consistent with findings seen in the colon and rectum[33]. Most authors recommend completion mucosectomy with pouch advancement for high-grade dysplasia and for recurrent or persistently positive biopsies of low-grade dysplasia[28,30]. Dysplastic changes may be a marker for mucosal instability and the risk of developing carcinoma in this setting should be eradicated.

CONCLUSION

Preserving the ATZ offers improved long-term function, clinical outcomes, and decreased postoperative complications and pouch failure; however, ATZ preservation carries a small risk for developing dysplasia or malignancy. As a result of the risk of developing dysplasia or cancer, a selective approach to stapled RPC IPAA should be undertaken, based on the presence of dysplasia, irrespective of the location and severity. Stapled RPC IPAA, and therefore preservation of the ATZ, should be reserved for those patients in whom multiple preoperative endoscopic biopsies rule out dysplasia or carcinoma in the entire colon. Transanal mucosectomy and handsewn IPAA should be performed in patients with biopsy-proven dysplasia, irrespective of the location and severity. Due to the significant inter-observer discrepancies noted, the presence of dysplasia should be confirmed by two independent pathologists. If these resection guidelines are utilized, it is believed that cancer risk can be further reduced, especially if postoperative endoscopic surveillance is employed.

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Morphological, kinetic, membrane biochemical and genetic aspects of intestinal enteroplasticity

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Abstract

The process of intestinal adaptation ("enteroplasticity") is complex and multifaceted. Although a number of trophic nutrients and non-nutritive factors have been identified in animal studies, successful, reproducible clinical trials in humans are awaited. Understanding mechanisms underlying this adaptive process may direct research toward strategies that maximize intestinal function and impart a true clinical benefit to patients with short bowel syndrome, or to persons in whom nutrient absorption needs to be maximized. In this review, we consider the morphological, kinetic and membrane biochemical aspects of enteroplasticity, focus on the importance of nutritional factors, provide an overview of the many hormones that may alter the adaptive process, and consider some of the possible molecular profiles. While most of the data is derived from rodent studies, wherever possible, the results of human studies of intestinal enteroplasticity are provided.

Key words: Diabetes; Diet; Hormonal regulation; Intestinal resection; Mechanisms; Morphology; Nutrient absorption; Short bowel syndrome; Signals

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INTRODUCTION

The intestine has an inherent ability to adapt morphologically and functionally in response to internal and external environmental stimuli. This process is called intestinal adaptation, or enteroplasticity. In fact, intestinal adaptation may be considered as a paradigm of gene-environment interactions. The array of phenotypic adaptations includes modification of brush border membrane (BBM) fluidity and permeability, as well as up- or down-regulation of carrier-mediated transport. Intestinal adaptation occurs following loss of a major portion of the small intestine (short bowel syndrome, SBS), following chronic ingestion of ethanol, following sublethal doses of abdominal irradiation, in diabetes, with aging, and with fasting and malnutrition[1-3].

Following intestinal resection, morphological and functional changes occur depending upon the extent and site of bowel removed, as well as external factors such as the lipid content of the diet (reviewed in Thiessen *et al*[4]). The increase in nutrient absorption from this process of enteroplasticity following resection compensates for the loss of mucosal absorptive surface area and minimizes the malabsorption that could otherwise occur. Therefore, intestinal adaptation has important implications in survival potential and welfare of the host[5]. In contrast, the adaptive process may be deleterious: for example, in diabetes this process enhances sugar and lipid uptake, exacerbating prevailing hyperglycemia, hyperlipidemia and obesity[6].

MECHANISMS

Mechanisms of intestinal adaptation occur at a variety of levels: physiological, cellular and molecular. Signals

of adaptation may relate to various hormone levels, transcription factors, ATP levels, or to changes in concentration of luminal solutes[3]. Signals for and mechanisms of the enteroplasticity process may be different for the jejunum and ileum, as well as in the intestinal crypt and villous tip, explaining site-specific alterations and differences between crypt and villous enterocytes[1-2].

Rodents are commonly used in well-characterized models for assessing the process of intestinal adaptation[7]. For example, following small bowel resection in the rat, the remnant intestinal mucosa undergoes compensatory alterations in an attempt to restore normal absorptive capacity[8]. Morphological and functional changes include increases in crypt depth and villous length, enterocyte proliferation, as well as increased electrolyte, glucose and amino acid uptake[7-8].

The adaptive process has been defined in terms of transport kinetics. Changes usually occur in the value of maximal transport rate (Vmax) rather than in Michaelis affinity (Km) constant of specific nutrient transporters (sugars and amino acids)[9-10]. Furthermore, there may be alterations in permeability coefficients of nutrients transported passively such as short-, medium- and long-chain fatty acids and cholesterol[1-2,11]. Increased Vmax results from either up-regulation of the total number of transporters per enterocyte, increased number of transporting mucosal cells, or increase in the intrinsic activity of the transporter[12-13]. Intestinal resection also selectively changes passive permeability properties of the BBM, as demonstrated by increased uptake of fatty acids, an increase that is not due simply to the changes in mucosal surface area or the effective resistance of the intestinal unstirred water layer (UWL)[14]. Indeed, this altered permeability is due to changes in the lipophilic properties of the BBM caused by variations in-lipid content of the BBM[15], which represents part of the adaptive process.

DIABETES AND INTESTINAL RESECTION AS EXAMPLES

Intestinal adaptation in the rodent model of chronic diabetes involves changes at the transcriptional as well as the post-transcriptional level, leading to increased Na^+-coupled sugar absorption[16]. After inducing acute hyperglycemia in rats, there is rapid up-regulation of glucose transport across the (enterocyte) basolateral membrane (BLM)[17]. In this model, both the vascular as well as luminal glucose infusion causes an increase in glucose transport capacity across the BLM[18]. No significant increase in BLM cytochalasin B binding or in GLUT2 protein abundance was observed, suggesting that there may be a post-translational event that increases the number of GLUT2 proteins available for transport, such as the movement of GLUT2 to the BLM from a preformed pool within the enterocyte. Alternatively, intrinsic activity of the transporter may be altered in the absence of changes in transporter protein abundance. Changes in intrinsic activity of glucose transporters have been observed with hyperglycemia[19], diabetes[20], low luminal glucose concentrations[12], and following activation of mitogen-activated protein kinase (MAPK) and P13K[13].

Following extensive intestinal resection, there is hyperplasia of the remaining small intestine, which is often accompanied by enhanced uptake of nutrients[21]. Alterations in the cell kinetics that result in modification of nutrition status may be specific or non-specific. Non-specific mechanisms involve alterations that result in changes in intestinal mucosal mass and/or villous surface area, leading to modifications in uptake of all nutrients, including those that are absorbed passively[22]. Specific mechanisms involve up- or down-regulation of transporters responsible for uptake of nutrients, such as sugars or amino acids[1-2].

THE IMPORTANCE OF MORPHOLOGY

The observation that morphological modifications may accompany intestinal adaptation in the rodent small bowel resection model was first made by Dowling and Booth[21]. The remaining intestine after resection is hyperplastic, with greater villous height and crypt depth, leading to enhanced mucosal surface area. While increased nutrient absorption is observed, the morphological changes do not necessarily solely explain alterations in nutrient uptake. For example, 1 wk after 80% small bowel resection, the remaining intestine increased its mass to 50%-70% of its pre-resection level, yet uptake of glucose increased only to approximately 33% of the pre-resection level[8].

It is clear that dynamic morphological parameters of the intestine may also adapt. For instance, crypt cell production rates or enterocyte migration rates along the villi change in some situations of intestinal adaptation[23]. It is important that morphological alterations be considered when estimating kinetic parameters of absorption. Morphological modifications such as blunting of mucosal growth or mucosal hyperplasia after intestinal resection are observed when dexamethasone is given subcutaneously[24]. Both transporter kinetics and dynamic morphological parameters are altered in the adaptive process, and the influence of resection on nutrient uptake is due to integration of these processes. This may be due to altered cell kinetics changing the population of enterocytes along the villus, thereby leading to variations in the number of cells with transporters, or activity of the transporters[25-26].

OTHER EXAMPLES

Many models of intestinal adaptation have been described: glucose uptake has been found to be increased during pregnancy[27], and lactation[28], with ingestion of a high carbohydrate diet[29], hyperglycemia[30], with diabetes[15], high alcohol intake[31] and after intestinal resection[32]. Glucose uptake is decreased with aging[33], external abdominal radiation[34], and with use of total parenteral nutrition[35]. Most transporters are up-regulated by levels of dietary substrate levels, and yet toxic substances and

essential amino acids have the opposite effect[7,35-37]. These examples illustrate the diversity and variability of this enteroplasticity process.

Increases in nutrient absorption have been documented[38-40] in humans following intestinal resection. The role of morphological changes in this process, however, has not been conclusively demonstrated. Remnant small bowel lengthening and dilatation has been noted in patients with SBS, suggesting morphological mechanisms in human intestinal adaptation[41]. However, the morphological adaptation typical in rodent models[21,42] (hypertrophy or hyperplasia) is not necessarily observed in the human adaptive response[43-44]. Several studies have shown no increase in villous height or crypt depth among patients who underwent intestinal resection, compared to healthy controls[39,45]. With the existence of various anatomical, physiological and biochemical differences between the human and rodent gastrointestinal tracts[46], and a conspicuous lack of comparable human studies, the clinical adequacy of the rat as a model of intestinal adaptation remains to be determined. Accordingly, caution must be used when attempting to extrapolate findings from rodent studies to the human population. Is there a better model? The neonatal piglet has been used in short bowel studies[47-49], and has been used to determine the effects of insulin-like growth factor-1 (IGF-l) and dietary manipulations[48,50]. The degree to which the results obtained using this model reflect human findings has yet to be determined, and the rodent remains a popular model for studies of intestinal adaptation.

DIETARY REGULATION

The topic of dietary regulation of intestinal gene expression has been reviewed[29,51]. Dietary constituents provide continual environmental signals that elicit expression of a host of genes that influence intestinal adaptation[52]. Every day, enterocytes are exposed to different nutrients that vary according to the nutrient intake of the host. For this reason, the intestine must be able to adapt to variations in the dietary load and composition[29,53]. The intestine, like many other biological and engineered systems, is quantitatively matched to prevailing peak loads with modest reserve capacities. Indeed, physiological capacities are optimal and most economical if they ascribe to the adage "enough, but not too much"[53]. Therefore, intestinal enzymes and transporters are characterized by a "safety factor", a parameter that represents the ratio of its capacity to the load placed on it[54]. The maintenance of this reserve capacity is biosynthetically costly, but is necessary given the unpredictable nature of dietary contents.

Let us consider the process of enteroplasticity and parenteral *vs* enteral nutrition, dietary lipids, carbohydrates, proteins and polyamines.

Parenteral vs enteral nutrition

In rodent models using total parenteral nutrition (TPN), small bowel atrophy is well characterized[55-57]. Not surprisingly, the presence of luminal nutrients also contributes greatly to enteroplasticity[58].

Dietary lipids

The dietary fat content influences the uptake of hexoses and lipids into rabbit jejunum following ileal resection[14]. In using a rat model of SBS, early feeding of a high-fat diet increased lipid absorptive capacity of the intestinal remnant[59]. A high-fat diet decreased mucosal mRNA levels of the lipid binding protein FAT/CD36, and decreased oleic acid uptake by isolated enterocytes. Mice that were chronically fed a diet enriched in sunflower oil had increased liver fatty acid binding protein (L-FABP) mRNA levels in the small intestine[60]. The effect was specific to this gene, as the intestinal fatty acid binding protein (I-FABP) was unaffected.

Not only the amount of fat, but also the type of dietary fat may influence intestinal function. Keelan *et al*[61] tested the hypothesis that intestinal morphology and uptake of nutrients after resection of the distal half of the small intestine of rats responds to alterations in dietary content of saturated (SFAs) and polyunsaturated (PUFAs) fatty acids. Adult female Sprague-Dawley rats were subjected to a sham operation or to surgical resection of the distal half of the small intestine. Animals were fed chow for 3 wk, then either chow or isocaloric semisynthetic SFA or PUFA diets for a further 2 wk. The *in vitro* jejunal uptake of glucose was twice as high in animals that had undergone resection and were fed SFAs than in those fed PUFAs. Perhaps SFAs are necessary in the diet to ensure that adequate adaptation takes place.

Thiesen and colleagues examined the effect of dietary lipids on lipid uptake in rats post-resection. Intestinal resection had no effect on mRNA expression of early response genes (ERGs), proglucagon or the ileal lipid binding protein (ILBP), but was associated with reduced jejunal mRNA for ornithine decarboxylase (ODC) and for L-FABP[62]. These resection-associated changes in gene expression were not linked with alterations in intestinal uptake of long-chain fatty acids or cholesterol. In animals undergoing intestinal resection and fed SFA, there was a reduction in jejunal proglucagon mRNA expression as compared to those animals fed chow or PUFA. ODC mRNA expression in the jejunum of resected animals was reduced. Thus, dietary lipids modify uptake of lipids in resected animals, and ODC and proglucagon may be involved in this adaptive response[63].

The way by which dietary lipids alter gene expression and consequently change membrane composition and/or nutrient transport may be through activation of peroxisome proliferator-activated receptors (PPARs), hepatic nuclear factor-4 (HNF-4), nuclear factor κB (NFKB), and sterol response element binding protein-lc (SREBP-1c)[52]. By binding to these transcriptional factors, dietary lipids affect the rate of transcription and consequently the protein synthesis of nutrient transporters[51,64]. PPARs belong to the super-family of receptors that include the glucocorticosteroid receptor[65].

When the locally acting glucocorticosteroid budesonide was administered concomitantly with an SFA diet, jejunal uptake of glucose was increased but ileal uptake of fructose was reduced[66].

It has been suggested that dietary lipids participate in signal transduction involving activation of second messengers, such as cAMP, Ca^{2+} and diacylglycerol, thereby changing the mRNA expression[67]. Studies with glycosphingolipid have revealed the importance of these lipids and their metabolites in signaling pathways *via* the tyrosine kinase-linked receptors. This is a signal system mediated by protein kinase C (PKC), MAPK, other kinases, as well as by cytosolic Ca^{2+} concentration[68]. Additional new signals involved in adaptive intestinal response 3 d after 50% intestinal resection have been identified by cDNA microarray analysis. These include proline-rich protein 2, involved in wound healing; glutathione reductase, a gene involved in intestinal apoptosis; NF-2 family members, also involved in apoptosis; etoposide-induced p53-mediated apoptosis; basic Kruppe-like factor, a transcription factor that activates the promoter for IGF-1; and prothymosin-α, involved in cell proliferation[69,70]. These observations of altered expression of signals are useful to generate hypotheses that can be tested in future studies to establish whether these signals represent a primary or a secondary event in enteroplasticity.

The glycospingolipid, phospholipid, cholesterol and fatty acid composition of plasma membranes may be modified in mammalian cells[71]. For example, Keelan *et al*[72] demonstrated that alterations in dietary fatty acid saturation influence intestinal BBM phospholipid fatty acid composition in rats. The investigators proposed that previously reported diet-associated changes in active and passive intestinal transport are due at least in part to these alterations in the fatty acid composition in BBM phospholipids. A diet enriched with SFA is associated with increases in the saturation of BBM phospholipid fatty acids, while a diet enriched with PUFA is associated with an increase in the unsaturation of BBM phospholipid fatty acids[1-2].

Meddings[73] compared *in vivo* membrane lipid permeability within the same intestinal region, under conditions where membrane physical properties were radically altered by feeding rats an inhibitor of cholesterol synthesis. Marked reductions in membrane fluidity were observed due to replacement of membrane cholesterol with its precursor, 7-dehydrocholesterol. Associated with these alterations was a pronounced reduction in membrane lipid permeability. Therefore, BBM membrane lipid permeability, *in vivo*, appears to be correlated with the physical properties of the bilayer.

The degree of fatty acid unsaturation or saturation, as well as the cholesterol and ganglioside/glycosphingolipid content, are factors that influence fluidity of the BBM[74-75]. Changes in fluidity of the BBM may alter passive permeation of molecules and nutrients through this barrier, as well as conformation of binding sites on transporter proteins such as SGLT1, GLUTS[71,76]. For example, alterations in BBM fluidity influence passive uptake of lipids, as well as carrier-mediated D-glucose uptake[76,77]. While enhancement of fluidity increases the uptake of lipids, fluidization of BBM from enterocytes located on the villous tip decreases uptake of D-glucose to levels seen in the BBM from enterocytes located in the crypts[78].

While altered membrane lipid composition may act in part by changing viscosity or fluidity of the BBM, it may also alter the microenvironment surrounding the transporter and thereby modify transporter activity. Two types of specialized microdomains in the BBM have been identified: lipid rafts and caveolae. These regions are important in signal transduction as well as lipid and protein trafficking[79-81]. Lipid rafts are enriched in SFAs, cholesterol and gangliosides[80-82].

Feeding rats a diet containing gangliosides increases jejunal glucose uptake[83]. Feeding them a ganglioside-rich diet increases ganglioside content and decreases cholesterol content in the intestinal mucosa, plasma, retina and brain[84]. Similar changes in lipid composition of intestinal microdomains, or lipid rafts, occur following ganglioside feeding[85]. Although SGLT1 has been localized to these microdomains in renal epithelial cells[86], it is not known if sugar transporters reside in intestinal BBM microdomains. If this is the case, local changes in membrane fatty acids may affect the activity of transporter by altering the configuration of the protein, potentially exposing or masking transporter binding sites and thereby modifying nutrient uptake. Gangliosides may also influence intestinal sugar transport *via* alterations on pro-inflammatory mediators, many of which are known to influence intestinal sugar transport[87-89]. For example, in rats challenged with lipopolysaccharide, ganglioside feeding reduced the production of intestinal platelet activating factor, PGE2 and LTB4, as well as plasma levels of IL-1β and TNF-α[90].

Dietary carbohydrates

Dietary carbohydrate may induce the intestinal adaptive response by increasing the abundance of hexose transporters to facilitate a higher rate of sugar absorption[9]. In a murine model, intestinal glucose uptake was directly correlated with dietary carbohydrate load[29,36,91]. The effect of dietary carbohydrate on nutrient transporter abundance has been reported in several animal models. For instance, abundance of SGLT-1 in BBM and GLUT2 in the BLM was elevated in animals fed a high carbohydrate diet; associated with this enhanced level of protein was an increase in glucose absorption[17,92-93]. The GLUT5 transporter abundance was also elevated with enhanced consumption of dietary fructose, leading to increased fructose uptake[77].

Initiation of dietary glucose-induced adaptive response occurs in the intestinal crypts, where transport capacities of nutrient transporters are programmed[91,36-37,93]. Utilizing a mouse model, diet from a high to a low carbohydrate regimen reduced the amount of glucose transporter, as estimated from density of phlorizin binding. Enterocytes may adapt to the high carbohydrate diet by increasing

crypt cell turnover rate, enhancing enterocyte migration rate, as well as by reprogramming the capability of nutrient transporters in the crypts to accommodate to the requirement for higher monosaccharide transporters[93]. Alteration in the density of glucose transporters was first observed in the crypt cells, and over a 3-d period was subsequently seen in the villous tip cells. Thus, crypt enterocytes respond to the high carbohydrate diet by increasing glucose transportation density. These cells then migrate up the villus over the next 3 d, contributing to the adaptation process enhancing glucose uptake.

Animals fed a glucose-enriched diet have increased glucose uptake, resulting from up-regulation of both BBM and BLM glucose transporters[17,92,94]. During early development, precocious introduction of dietary fructose causes enhanced expression of fructose transporters and fructose transport, without changing glucose uptake[93]. The substrates glucose and fructose specifically up-regulate their own transporters, SGLT-1 and GLUT5. In contrast, increases in essential amino acids or other substances that are potentially toxic at high levels (such as iron, calcium or phosphorous) are associated with no changes, or even reductions, in transport[35,95].

In many cases, other nutrients may be equal, or even more potent, inducers of the transporter than the transporter's specific substrate. For example, young animals fed a diet enriched with PUFA have a decline in glucose uptake, as compared to animals fed an SFA-enriched diet[66,96,97]. Similarly, Vine *et al*[98] studied the effect of varying fatty acids on the passive and active transport properties of rat jejunum and found that an SFA-enriched diet increased Na^+-dependent glucose uptake when compared to a diet enriched with n6 PUFA. In contrast, in aged rats, glucose uptake is increased by PUFA and not by SFA[33].

Dietary fiber also modulates intestinal nutrient uptake. For example, in dogs, a diet enriched with fermentable fiber increases glucose uptake and GLUT2 transporter abundance[99]. *In vitro* studies, in which rat intestinal tissue was incubated with B-glucan isolated from barley or oats, show reductions in uptake of stearic and linoleic acids (Drozdowski *et al*, 2005, unpublished observations). Furthermore, many studies have investigated the effect of TPN supplemented with short-chain fatty acids, the products of fiber fermentation. Increases in glucose uptake, GLUT2 mRNA and protein, and intestinal morphology were observed in normal rats as well as in rats following intestinal resection[100-103].

Dietary proteins

Dietary proteins also have an impact on intestinal morphology and active amino acid transport[37,104]. Both *in vitro*[105] and *in vivo*[104] rat experiments have shown that a high-protein diet increases amino acid uptake in the jejunum. Alteration in the amount of dietary protein induces reversible adaptation of non-essential amino acid transport rate[106]. Feeding a high-protein diet to mice induces a 77%-81% increment in uptake of non-essential amino acids[35], yet only a 32%-61% increase for essential amino acids. A protein-deficient regimen reduces uptake of non-essential amino acids, such as aspartate and proline, and maintains or increases uptake for essential amino acids and alanine.

Glutamine is a key metabolic fuel for enterocytes, mediating cellular nucleic acid synthesis and proliferation. Parenterally fed rats demonstrate decreased atrophy of the intestinal mucosa following glutamine supplementation[106]. Glutamine administration also normalizes the reduced levels of intestinal adaptation in rats receiving TPN following intestinal resection[107]. It is noteworthy that some studies of oral glutamine supplementation in the rat have failed to document more than temporary mucosal proliferation[108].

Other amino acids may inhibit intestinal adaptation. Sukhotnik *et al*[109] examined effects of parenteral administration of the nitric oxide precursor arginine to rats following 75% small bowel resection. Arginine supplementation was associated with lower cell proliferation and greater enterocyte apoptosis. Thus, the nature of the adaptive response depends upon the type of amino acid and the needs of the animal.

Polyamines

Polyamines are found in all eukaryotic cells[110], and they play an important role in growth and differentiation[111]. Polyamines are obtained either from the diet, or *via* synthesis from ornithine[112]. Luminal perfusions of polyamines rapidly (in less than 5 min) enhance intestinal glucose uptake in rats and increase BBM SGLT-1 protein[113].

Polyamine synthesis or uptake may be an important event that initiates adaptive hyperplasia observed in the intestinal remnant after partial small bowel resection. Enteral diets supplemented with ornithine alpha-ketoglutarate (OKG), a precursor for arginine, glutamine and polyamines, enhances intestinal adaptation in models of intestinal resection[114,115]. Indeed, studies by both Tappenden *et al*[116] and Thiesen *et al*[62] suggest that ODC, a key enzyme in polyamine synthesis, may mediate the adaptive process in rats that is stimulated by the administration of either glucocorticosteroids or short-chain fatty acids to rats following intestinal resection.

HORMONAL REGULATION

Glucocorticosteroids (GCs)

Numerous hormones modify the form and function of the intestine. It is not clear whether any of the dietary features that modify the intestinal adaptive process do so by way of hormonal alterations. In a model of extensive intestinal resection (50% enterectomy), the remaining proximal and distal intestinal remnants were adequate to assess the morphology and function at these sites[9,106]. The GC prednisone had no effect on intestinal uptake of glucose or fructose in these resected animals[62]. In contrast, the locally acting steroid budesonide increased by over 120% the value of the jejunal Vmax for the uptake of glucose, and increased by over 150% the ileal uptake of fructose. The protein abundance and mRNA

expression of SGLT-1, GLUTS, GLUT2 and Na^+/K^+ APTase $\alpha 1$ and $\beta 1$ did not explain this enhancing effect of budesonide on glucose and fructose uptake. Budesonide, prednisone and dexamethasone reduced jejunal expression of the early response gene c-jun. In resected animals, the abundance of the mRNA of ODC in the jejunum was reduced, and GCs reduced jejunal expression of mRNA for proglucagon. These data suggest that enhancing influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC and proglucagon, or other as yet unknown signals.

In contrast, the uptake of D-fructose by GLUT5 was similarly increased with budesonide and with prednisone. Increases in the uptake of fructose was not due to variations in weight of intestinal mucosa, food intake, or in GLUT5 protein or mRNA expression. There were no steroid-associated changes in mRNA expression of c-myc, c jun, c-fos, of proglucagon, or of selected cytokines. However, the abundance of ileal ODC mRNA was increased with prednisone. Giving post-weaning rats budesonide or prednisone in 4-wk doses equivalent to those used in clinical practice increases fructose but not glucose uptake. This enhanced uptake of fructose was likely regulated by post-translational processes[62].

Growth hormone (GH)

GH has been suggested as possessing pro-adaptive properties[117]. In rats and piglets, GH administration results in an increase in small bowel length and function per unit length[118]. Hypophysectomized rats undergo mucosal hypoplasia of the small bowel, as well as a reduced adaptive response following resection that is restored by GH[119]. In contrast, transgenic mice expressing elevated levels of GH experience hypertrophy of the small intestine[118]. IGF-1 expression in the small bowel is regulated by GH and is believed to induce enterotrophic effects following resection[120,121]. In a rat model of SBS, acute IGF-1 treatment of TPN-fed rats produced sustained jejunal hyperplasia, and facilitated weaning from parenteral to enteral nutrition[122]. GH administration to normal rats has been reported to have positive effects on mucosal growth and intestinal adaptation following massive resection[123], although contradictory data exist[124,125]. Human and rabbit studies have indicated that increased nutrient transporter activity devoid of morphological changes may be the method of GH-induced intestinal adaptation[126].

GH administration inhibits liberation of glutamine from muscle during catabolic states in humans[127]. This suggests a possible role for combining GH and glutamine to achieve optimal adaptation. Trials investigating any such synergism in the rat have yielded conflicting results. Some studies have failed to demonstrate an additive effect of GH and glutamine in the enhancement of post-resection intestinal adaptation[128], while others have documented a positive synergistic effect[129]. For example, GH has been shown to enhance the absorption of amino acids using *ex vivo* human BBM vesicles[129]. An intestinal mucosal GH receptor has been described in rats and humans[130], and GH promotes cell differentiation and clonal expansion of these differentiated cells[131].

Human studies have suggested that the efficacy of GH and/or glutamine therapy in the adaptive response of the small bowel may be based heavily upon the clinical status of the patient[132]. Evaluation of the effect of such may facilitate further understanding of the pathology and physiology of the bowel adaptation process, as well as more clearly defining positive predictive indicators of the bowel's ability to be rehabilitated. Existing human data indicate that administration of high concentrations of GH can actually increase patient morbidity and mortality[133].

In studies of home parenteral nutrition (HPN)-dependent patients with SBS, the use of high-dose recombinant human GH (0.4 mg/kg per day) in controlled[133,134] and uncontrolled studies[135] has led to variable results. Patients were given glutamine supplements by mouth or parenterally, and their diet was modified. In the randomized, placebo-controlled study of Scolapio *et al*[133], subjects ingested a standardized 1500 kcal/d diet, which is clearly different from the hyperphagic diet consumed by many SBS patients[136], and which may contribute to the physiological adaptation that occurs in the remaining intestine after extensive resection. It is unclear whether glutamine is beneficial for the adaptive response in humans. In rat models of SBS, it is unclear whether glutamine supplementation is efficacious for the adaptive process[137-138]. Furthermore, both a hyperphagic diet and absence of malnutrition are needed for humans to achieve optimal intestinal adaptation[41,139].

When HPN-dependent patients with SBS were provided a usual *ad libitum* hyperphagic diet, and given low doses of GH (0.05 mg/kg per day) for 3 wk, there was significant improvement in intestinal absorption of energy (15% ± 5%), nitrogen (14% ± 6%) and carbohydrate (10% ± 4%)[140]. Increased food absorption represented 37% ± 16% of total parenteral energy delivery. Body weight, lean body mass, D-xylose absorption, IGF-1, and IGF binding protein 3 increased, whereas uptake of GH binding protein decreased. During treatment with GH, improvement in net intestinal absorption compared with placebo was 427 ± 87 kcal/d, representing 19% ± 8% of the total energy expenditure required to obtain energy balance equilibrium in patients with SBS[136].

From a review of literature in this area, Matarese *et al*[140] noted that there were differences in gastrointestinal (GI) anatomy, dietary compliance, nutritional status, presence of mucosal disease, and diagnosis both within and between studies. These authors concluded that "administering recombinant human growth hormone alone or together with glutamine with or without a modified diet may be of benefit when the appropriate patients are selected for treatment".

IGF-1

IGF-1 proved to be efficient in increasing intestinal adaptation following resection in rats. IGF-l treatment

following 70% jejuno-ileal resection attenuated fat and amino acid malabsorption[141] and increased total gut weight by up to 21%. The IGF-1 receptor was increased in the jejunum and colon due to resection. Resection also increased circulating IGF-binding proteins (IGFBPs). IGF-1 treatment had no effect on IGF-1 mRNA or IGF-1 receptor density, but increased IGFBP-5 in the jejunum[142]. This increase in IGFBP-5 was correlated with jejunal growth after IGF-1 treatment[142].

IGF-1 treatment in resected rats significantly increased jejunal mucosal mass by 20% and mucosal concentrations of protein and DNA by 36 and 33%, respectively, above the response to resection alone[143]. These changes reflected an increase in enterocyte proliferation and an expansion of the proliferative compartment in the crypt. No further decrease in enterocyte apoptosis, or increase in enterocyte migration[144].

IGF-1 treatment may also facilitate weaning from parenteral to enteral nutrition. After 60% jejunoileal resection plus cecectomy, rats treated with recombinant human IGF-1 (3 mg/kg body weight per day) or control vehicle were maintained exclusively with TPN for 4 d, and were then transitioned to oral feeding. TPN and IGF-1 were stopped 7 d after resection, and rats were maintained with oral feeding for 10 more days. Acute IGF-1 treatment induced sustained jejunal hyperplasia, as suggested from the presence of greater concentrations of both jejunal mucosal protein and DNA, and was associated with maintenance of a greater body weight and serum IGF-1 concentrations[122].

Using male transgenic mice with targeted smooth muscle IGF-1 over-expression[145], these as well as non-transgenic littermates underwent 50% proximal small bowel resection. Growth factor over-expression led to a unique mucosal response characterized by a persistent increase in remnant intestinal length and an increase in mucosal surface area. Therefore, IGF-1 signaling from within the muscle layer may be important in resection-induced intestinal adaptation. In summary, IGF-1 shows promise as a hormone which may prove to be of clinical significance in nutritional regulation and the modification of intestinal absorption in the short and long term[138].

Epidermal growth factor (EGF)

EGF up-regulates intestinal nutrient transport[146]. This effect is mediated by PKC and P13K[147], and involves redistribution of SGLT-1 from microsomal pools to the BBM[148,149]. After massive intestinal resection, endogenous EGF is increased in saliva and is decreased in urine[150]. EGF stimulates intestinal adaptation after intestinal resection: the BBM surface area and total absorptive area increased until day 10, and EGF treatment induced a further increase in BBM surface area[151]. In a study by O'Brien and colleagues[152], mice underwent 50% small bowel resection or sham operation, and were then given orally an EGF receptor (EGFR) inhibitor (ZD1839, 50 mg/kg per day) or control vehicle for 3 d. ZD1839 prevented EGFR activation, as well as the normal postresection increases in ileal wet weight, villus height, and crypt depth. Enterocyte proliferation was reduced two-fold in the resection group by ZD1839. These results more directly confirm the requirement of a functional EGFR as a mediator of postresection adaptation response. Previous work has demonstrated that the EGFR is predominantly located on the BLM of enterocytes[153], but after small bowel resection the EGFR shows redistribution from the BLM to the BBM, with no change in the total amount of EGFR[154]. It is not known how this redistribution occurs. This is an important point, since modification of this process may represent a useful means to accelerate the intestinal adaptive process.

Laser capture microdissection (LCM) microscopy was used to elucidate the specific cellular compartment(s) responsible for postresection changes in EGFR expression[155]. Mice underwent a 50% proximal resection or sham operation, and after 3 d, frozen sections were taken from the remnant ileum. Individual cells from the villi, crypt, muscularis and mesenchymal compartments were isolated. EGFR mRNA expression for each cell compartment was quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR). EGFR expression was increased two-fold in the crypts after resection. This supports the hypothesis that EGFR signaling is crucial for the mitogenic stimulus for adaptation. The additional finding of increased EGFR expression in the muscular compartment is novel and may imply a role for EGFR in the muscular hyperplasia seen after massive small bowel resection. As noted previously, it is of interest that the muscle layer also appears to play a role in the adaptive response to IGF-1[155].

Treatment of resected rats with EGF has been studied: male juvenile rats underwent either transection or ileocecal resection leaving a 20-cm jejunal remnant[156]. Resected animals were treated orally with placebo or recombinant human EGF. Resected EGF-treated animals lost significantly less weight than those in the transection group, absorbed significantly more 3-0 methylglucose, and had reduced intestinal permeability as determined by the lactulose/mannitol ratio. Work by Chung *et al*[149] using rabbits showed that intestinal resection altered SGLT-1 mRNA and protein expression along the crypt-villous axis, with expression being highest in the mid-villous region. Oral EGF normalized SGLT-1 expression, resulting in a gradient of increasing expression from the base of the villous to the villous tip.

Nakai and colleagues[157] investigated the role of EGF in stimulating intestinal adaptation following small bowel transplantation. Treatment of rats with EGF (intraperitoneally for 3 d) following intestinal transplantation resulted in increased glucose absorption, SGLT-1 abundance and villous height and crypt depth in the graft. Clearly, there are sufficient animal data to support studies of the potential pro-adaptive role of EGF in humans.

Keratinocyte growth factor (KGF)

In a study by Yang *et al*[158], adult C57BL/6J mice were randomized to 55% mid-small bowel resection, resection with KGF administration, or a sham-operated (control)

group, and were killed at day 7. Ussing chamber studies showed that KGF increased net transepithelial absorption of 3-0-methyl glucose as well as sodium-coupled alanine absorption, but had no effect on epithelial permeability barrier function. Epithelial cells were separated along the crypt-villous axis with LCM, and epithelial KGF receptor (KGFR) mRNA abundance was studied using real time RT-PCR. KGF up-regulated KGFR mRNA abundance, predominately in the crypt and the lower portion of the villus.

Leptin and ghrelin

Leptin plays an important role in the regulation of body fat and satiety (reviewed in Jequier[159]). Leptin reduces food intake[160] and leptin-deficient mice develop obesity[161]. Leptin may be a potential growth factor for the normal rat small intestine. The effect of 14 d of parenteral leptin administration (2 μg/kg per day) to rats following 80% small bowel resection was studied. Leptin was associated with a 44% increase in galactose absorption and a 14% increase in GLUT-5 abundance, but with no change in DNA content or in SGLT abundance. These findings suggest that leptin may potentially be clinically useful in patients with impaired intestinal function[162].

Ghrelin is a gastric hormone that is released in response to enteral nutrients. It has an opposite effect when compared to leptin, as it stimulates food intake[163]. The role of ghrelin in intestinal adaptation is unknown.

Glucagon-like peptide 2 (GLP-2)

Animal studies have demonstrated a potential role for GLP-2 in the adaptive response following intestinal resection[143]. Plasma GLP-2 levels rise following intestinal resection in rats[164-166]. In a study by Dahly *et al*[143], rats were subjected to 70% mid-jejunoileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP-2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats. While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 d post-resection. These data support a significant role for endogenous GLP-2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats[167].

Correlations between post-resection GLP-2 levels, morphological indices, crypt cell proliferation rates and nutrient absorption have been made[168]: an inverse correlation was found between post-prandial GLP-2 levels and fat or protein absorption, as assessed by a 48-h balance study. These results, along with data obtained from studies showing that GLP-2 immunoneutralization inhibits post-resection adaptation[169], further implicate GLP-2 as a post-resection mediator of adaptation.

GLP-2 administration in rats increases the adaptive response to massive intestinal resection[170]. In this study, Sprague-Dawley rats were divided into two groups, one with a 75% mid-jejunum-ileum resection, and a second sham operated group. Animals were treated with 0.1 pg/g GLP-2 analog (protease resistant human GLP-2) or placebo given subcutaneously twice daily for 21 d. The groups were compared measuring the total weight of the rats, and mucosal mass per centimeter. Administration of GLP-2 or its analogs was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum. While resection reduced D-xylose excretion, GLP-2 restored D-xylose excretion to levels above control values within 21 d of administration. This indicates that GLP-2 has a positive effect on intestinal morphology and absorptive function following resection.

Martin *et al*[170] investigated the effects of GLP-2 in a TPN-supported model of experimental SBS. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, or TPN + 10 μg/kg per hour GLP-2. TPN plus GLP-2 treatment resulted in increased bowel and body weight, villous height, intestinal mucosal surface area, and crypt cell proliferation. GLP-2 reduced the lactulose-mannitol ratio, indicating that GLP-2 lowered intestinal permeability when compared with the TPN alone. GLP-2 increased serum GLP-2 levels as well as intestinal SGLT-1 protein abundance compared with either TPN or enteral groups. This study demonstrates that GLP-2 is capable of stimulating intestinal adaptation in the absence of enteral feeding.

Since a number of hormones and growth factors have been shown to influence intestinal function, Washizawa *et al*[171] compared the effects of GLP-2, GH and KGF on markers of gut adaptation following massive small bowel resection. KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in the small bowel and the colon. While both GH and KGF increased colonic mucosal growth, GLP-2 exerted superior trophic effects on jejunal growth. GLP-2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP-2, GH and KGF on gut adaptation following massive small bowel resection, the authors conclude that a combination of these agents may be most beneficial.

A pilot study to determine efficacy of GLP-2 in patients with SBS has been completed. A non-placebo controlled study was conducted in eight patients with SBS with an end-enterostomy type of anastomosis (six had Crohn's disease and four were not receiving HPN)[172]. Treatment with GLP-2 (400 μg subcutaneously twice a day for 35 d) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in five and six patients, respectively.

A review by Jeppesen[173] on the role of GLP-2 in treatment of SBS concludes that: "Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research

studies"[11]. The optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of parenteral nutrition in a larger fraction of short-bowel patients.

OTHER POSSIBLE SIGNALS OF INTESTINAL ADAPTATION

Dodson *et al*[174] identified three subsets of genes that were up-regulated by constructing a cDNA library from the remnant ileum of resected rats. This library was screened, and subtractive hybridization was used to identify genes that were induced following resection. These included genes involved with regulating the absorption and metabolism of nutrients. For example, L-FABP, apolipoprotein A-IV, cellular retinal binding protein II and ileal lipid binding protein were identified as genes that were induced following 70% intestinal resection in rats. Genes involved in cell cycle regulation were also identified. For example, phosphorylation and dephosphorylation are important regulators of the cell cycle, and PP1S, a subunit of a serine/threonine phosphatase was indeed up-regulated. Grp78, a member of the heat shock protein family was also increased. Grp78 resides in the endoplasmic reticulum and acts as a chaperone during protein assembly and transport. It may also have a protective role, and prevent apoptosis as a way of promoting the proliferative response following intestinal resection[175-176].

Rubin *et al*[177] further characterized the molecular and cellular mechanisms following 70% resection in rats. An immediate early gene, PC4/TIS7, was markedly increased soon after resection, with levels returning back to normal by 1 wk post-resection. Although the function of this protein is unknown, it may be related to cytodifferentiation as it is expressed only in the villus and not in the crypts.

Erwin *et al*[70] used cDNA microarrays to gain insight into the mechanism of intestinal adaptation. Mice underwent a 50% intestinal resection, and 3 d afterwards RNA was extracted from the remnant ileum. Multiple genes were induced, and fall into four categories: (1) apoptosis, DNA synthesis, repair and recombination (ten genes); (2) oncogenes, tumor suppressors, cell cycle regulators (three genes); (3) stress response, ion channels and transport (four genes); (4) transcription factors and general DNA-binding proteins (one gene).

Many of the genes (ODC, c-neu, glucose-related protein, IGFBP-4) that were identified agreed with the results of other studies of intestinal resection. For example, ODC was increased in this study, and this agrees with previous findings that showed ODC to be involved in the adaptive process[60,116,178]. Some new factors were also identified including glutathione reductase (involved in apoptosis), basic Kruppel-like factor (transcriptional regulator that activates the IGF promoter), prothymosin-α (associated with increased cell proliferation), and eteposide-induced p53-responsive mRNA (stress response protein involved in p53 mediated apoptosis).

Stern *et al*[69] performed a similar analysis of gene expression following 50% intestinal resection in rats. The gene with the largest increase was identified as Sprr2, a novel gene not previously known to be involved in intestinal adaptation. EGF administration post-resection further increased sprr2 expression, and enhanced the adaptive response. This protein plays a role in terminal differentiation of stratified squamous epithelium. Its role in the intestinal epithelium is unclear and warrants further investigation.

Finally, a variety of other signals have been described as possibly playing a role in the process of intestinal adaptation. These include prostanoids[179], uncoupling proteins[180], PPAR-α, transforming growth factor-α[181], SPARC (secreted protein, acidic and rich in cysteine[182], Bcl-2[183], endothelin-1[184], erythropoietin[185], the GATA family of zinc finger transcription factors[186], hepatocyte growth factor[187], the ERGs[188], PC4/TIS7[177] and epimorphin[189]. Augmented Wnt signaling has been shown to enhance the adaptive response to massive small bowel resection[190]. Several of these signals may be useful to modify in a clinical setting to enhance the intestinal adaptive response.

Microarray technology is a powerful tool that is constantly developing into a more sophisticated technique of identifying novel genes involved in physiological processes. Intestinal adaptation awaits further characterization by hypothesis-testing studies. From the information that is available at this time, it is clear that genes regulating the cell cycle, proliferation, differentiation and apoptosis are important components of the adaptive process, leading to enteroplasticity.

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REVIEW

Epidemiology and gene markers of ulcerative colitis in the Chinese

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Abstract

Inflammatory bowel disease (IBD) includes two similar yet distinct conditions called ulcerative colitis (UC) and Crohn's disease (CD). These diseases affect the digestive system and cause the inflammation of intestinal tissue, form sores and bleed easily. Most children with IBD are diagnosed in late childhood and adolescence. However, both UC and CD have been reported as early as in infancy. Most information pertaining to the epidemiology of IBD is based upon adult studies. Symptoms include abdominal pain, cramping, fatigue and diarrhea. Genetic factors play a significant role in determining IBD susceptibility. Epidemiological data support a genetic contribution to the pathogenesis of IBD. Recently, numerous new genes have been identified as being involved in the genetic susceptibility to IBD: *TNF-308A*, *CARD15* (*NOD2*), *MIF-173*, N-acetyltransferase 2 (*NAT2*), *NKG2D* (natural killer cell 2D), *STAT6* (signal transducer and activator of transcription 6), *CTLA-4* (cytotoxic T lymphocyte antigen-4), *MICA-MICB* (major histocompatibility complex A and B), *HLA-DRB1*, *HLA class-Ⅱ*, *IL-18*, *IL-4*, *MICA-A5*, *CD14*, *TLR4*, *Fas-670*, *p53* and *NF-κB*. The characterization of these novel genes has the potential to identify therapeutic agents and aid clinical assessment of phenotype and prognosis in patients with IBD (UC and CD).

Key words: Genetic; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Epidemiology; Susceptibility; Gene

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two primary types of inflammatory bowel disease (IBD). These two diseases have many similarities and sometimes are difficult to distinguish from each other. However, there are several differences. UC is an inflammatory destructive disease of the large intestine characterized by motility and secretion disorders. Inflammation usually occurs in the rectum and lower part of the colon, but it may affect the entire colon. UC rarely affects the small intestine except for the end section, called the terminal ileum. UC may also be called colitis or proctitis. Inflammation frequently makes the colon empty, causing diarrhea. Ulcers formed in places where the inflammation has killed the colon cells cause, bleeding and pus discharge. UC is an IBD that causes inflammation in the small intestine and colon. UC is difficult to diagnose because its symptoms are similar to other intestinal disorders and another type of IBD called CD. CD differs from UC because it causes deeper inflammation within the intestinal wall. CD usually occurs in the small intestine, although it can also occur in the mouth, esophagus, stomach, duodenum, large intestine, appendix, and anus. UC may occur in people of any age, but most often it starts between the ages of 15 and 30 years, or less frequently between 50 and 70. Children and adolescents sometimes develop the disease. UC affects men and women equally and appears to occur in some families. Clinical and epidemiological data do not support a simple Mendelian model of inheritance for IBD. CD and UC are considered to be complex

polygenic diseases. UC is a chronic disease in which the large intestine becomes inflamed and ulcerated (pitted or eroded), leading to flare-ups (bouts or attacks) of bloody diarrhea, abdominal cramps, and fever. The long-term risk of colon cancer is increased[1-3].

To make a diagnosis of IBD, physicians must first exclude other possible causes of inflammation. For example, infection with parasites or bacteria may also cause inflammation. Therefore, several tests should be performed. Stool samples are analyzed for evidence of a bacterial or parasitic infection (e.g. acquired during travel), including a type of bacterial infection (*Clostridium difficile* infection) that can result from antibiotic use, and sexually transmitted diseases of the rectum, such as gonorrhea, herpes virus infection, and chlamydia infection. Tissue samples (biopsies) may be taken from the lining of the rectum during sigmoidoscopy (an examination of the sigmoid colon using a viewing tube) and examined microscopically for evidence of other causes of colon inflammation (colitis). Other possible causes of similar abdominal symptoms that should be excluded are ischemic colitis, which occurs more often in people older than 50 years; certain gynecological disorders; celiac disease; and irritable bowel syndrome[1].

In traditional Chinese medicine (TCM), UC is a systemic disease that affects many parts of the body, although patients mainly manifest with intestinal symptoms. In TCM principles, the problem is closely associated with organ dysfunction, in particular the *pi* (spleen), that causes a failure to self-regulate the intestinal environment. TCM specialists generally agree that constitutional weakness, invasion of exogenous pathogens, an unbalanced diet and emotional factors all contribute to the development of the problem.

Genetic changes of patients with UC have been demonstrated. What are the changes in Chinese UC patients? There are gene diversification analyses of Chinese patients with UC in the recent literature.

ETIOLOGY AND EPIDEMIOLOGY

Etiology

The etiology of UC is unknown. The consensus is, so far, that it is a response to environmental triggers (infection, drugs or other agents) in genetically susceptible individuals. The genetic component is not as strong in UC as in CD. However, 10%-20% of patients with UC have at least one family member with IBD[1-2]. There are marked differences between ethnic groups and some (such as Ashkenazi Jews) have a particularly high incidence. Non-steroidal anti-inflammatory drugs may cause an episode of acute active disease in some patients with IBD. UC primarily affects young adults, but it can occur at any age from 5 to 80 years and women tend to be more commonly affected than men. It is a worldwide disorder, and the high-incidence areas include the United Kingdom, the United States, northern Europe and Australia. Low-incidence areas include Asia, Japan, and South America. The causes of UC remain unknown. The major theories are associated with infection, allergy to food component, genetics, environmental factors, and immune response to bacteria or other antigens[1].

Typical symptoms during flare-ups include abdominal cramps, an urge to move the bowels, and diarrhea (typically bloody). The diagnosis is based on an examination of the sigmoid colon using a flexible viewing tube (sigmoidoscopy) or an examination of the large intestine using a flexible viewing tube (colonoscopy). People who have had UC for a long time may develop colon cancer. Treatment is aimed at controlling the inflammation, reducing symptoms, and replacing any lost fluids and nutrients. UC may start at any age but usually begins between the ages of 15 and 30 years. A small group of people have their first attack between the ages of 50 and 70 years. UC usually does not affect the full thickness of the wall of the large intestine and hardly ever affects the small intestine. The disease usually begins in the rectum or the rectum and the sigmoid colon (the lower end of the large intestine) but may eventually spread along part or all of the large intestine. UC, which is confined to the rectum, is a very common and relatively benign form. In some people, most of the large intestine is affected early.

Epidemiology

UC affects about one in 1000 people in the Western world. Peak incidence is between the ages of 10 and 40 years. UC may affect people of any age and 15% of people are over the age of 60 years at diagnosis[1-4]. The incidence of UC in North America is 10-12 cases per 100 000, with a peak incidence of UC occurring between the ages of 15 and 25 years. There is thought to be a bimodal distribution in age of onset, with a second peak in incidence occurring in the sixth decade of life. The disease affects females more than males, with highest incidences in the United States, Canada, the United Kingdom, and Scandinavia. Higher incidences are seen in the northern parts compared to the south in Europe and the United States.

Rising incidence and prevalence of UC have been observed in Asian countries. Lok *et al*[2] conducted a study in an Asian center, aiming to describe the epidemiology and clinical characteristics of UC in the local Chinese population. This is a retrospective analysis of patients with diagnosis of UC in our hospital from June 1990 to December 2006. The diagnosis of UC has to satisfy the internationally accepted criteria. All patients were Chinese residents in a well-defined catchments' area. Clinical and epidemiological data were obtained from medical records and patient interviews. Seventy-three Chinese UC patients were managed in hospital. The hospital-based prevalence has risen by three times over a 10-year period, but no definite rising incidence can be demonstrated. The mean age at diagnosis was 40.6 years and the median duration of disease was 72 mo. In the patient cohort, 38.4% had ulcerative proctitis and 26% had left-sided UC, whereas 35.6% had extensive UC at presentation. The majority presented with mild (39.7%) or moderate (30.2%) disease activity, but 27.4% presented with severe disease. One of the two patients (2.7%) with fulminant disease developed

toxic megacolon. Extra-gastrointestinal manifestations occurred in 13.7%. During the follow-up period, most patients (86.3%) were in disease remission. Four patients (5.5%) underwent colectomy, four (5.5%) died, and two (2.7%) were lost to follow-up. The prevalence but not the incidence of UC is rising in the Chinese population. It usually affects young patients and a substantial proportion of patients presented with severe and fulminant disease. The disease activity of most Chinese patients can be controlled with medical treatment, though a small proportion of patients need surgery or have a fatal outcome[2-4].

Jin *et al*[3] explored the indications for colonoscopic examination and the distribution of diagnostic diseases. From January 2000 to December 2004, 5960 patients received colonoscopic examination in a colorectal center. The indications for colonoscopic examination and the distribution of its diagnostic diseases were analyzed. There were 3096 males and 2594 females, and the mean age was 52 ± 15 years. The reasons for colonoscopy included hemafecia (26.9%), atypical abdominal pain (25.8%), diarrhea or increased frequency of stools (11.1%), anal tenesmus or discomfort (7.6%), constipation (7.0%), mucous or bloody purulent stools (3.0%), intra-rectal mass or abdominal mass on physical examination (0.9%), re-examination after colonoscopic polypectomy (10.9%), re-examination after operation for colorectal cancer (1.5%), and simple health examination (2.2%). Colonoscopy reached the cecum in 97.7% of the cases, and at least one disease was found in 2283 cases (40.1%). Among them, colorectal cancer accounted for 10.3%, colorectal polyps 19.6%, UC 4.3%, and CD 0.5%. The indications for colonoscopy are too strict to screen for early stage colorectal cancer. Colonoscopy should be performed in patients with symptoms such as bloody stools, diarrhea, abdominal pain, constipation, or with colorectal polyps, after operation for colorectal cancer, or as members of a hereditary colorectal cancer family[3]. Cigarette smoking, alcohol use, appendectomy, and family history of IBD have all been shown to be associated with IBD, but there was no report of risk factors for IBD in a Chinese population, in which the incidence of IBD has increased during the past decade. Jiang *et al*[4] conducted a case-control study to examine associations between previously reported environmental risk factors and development of UC in Wuhan city, central China. A total of 177 patients with UC and 177 age-matched and sex-matched controls were prospectively studied in Wuhan city from January 2004 to December 2004. An age-matched and sex-matched case-control study was conducted to assess the role of smoking, alcohol use, appendectomy, and other potential risk factors in the development of UC, by a detailed questionnaire. Smoking was a protective factor and ex-smoking is a risk factor for UC (compared with non-smokers, smokers: $P = 0.0001$; ex-smokers: $P = 0.008$). Positive family history of IBD was a risk factor ($P = 0.025$), whereas appendectomy was a protective factor ($P = 0.028$) for UC. There were no significant associations between UC and other factors examined. Although the incidence of UC in Chinese is relatively lower than that in whites, the same risk factors for UC that were reported in white populations were associated with Chinese UC patients. Specifically, smoking was a protective factor for UC and ex-smoking was associated with an increase risk of UC in a Chinese population. Family history of IBD was shown to be a risk for UC, whereas appendectomy was associated with a low risk for UC[2,4].

Investigative papers about IBD in Chinese medical journals from 1989 to 2003 were reviewed to understand the progress of basic IBD research in China, by Bai *et al*[5]. The basic science investigative papers about IBD from 1989 to 2003 in Chinese periodicals (VIP and CMCC) were reviewed and analyzed; the key words used were as follows: IBD, UC, CD, basic science investigation, and literature review. There were 3454 articles about IBD published in Chinese medical journals from 1989 to 2003, and during these 15 years, 508 papers focused on basic scientific investigations. There were 463 papers investigating the pathogenesis of IBD, 287 papers on immunological mechanisms, and 176 papers about other mechanisms. There were 142 papers investigating the mechanisms of TCM in IBD from 1989 to 2003, which included 117 papers related to animal experiments and 25 papers related to clinical studies. There have been relatively few investigative scientific papers on IBD published in Chinese medical journals. However, the study of IBD has been emphasized in China. Research on the immunological mechanisms of IBD has been predominant. Furthermore, a large number of the research papers are about the mechanisms and effects of TCM in IBD.

IBD had been uncommon in China until about 1990, but since then, more and more cases have been seen in clinical settings. The prevalence and phenotype of IBD in the Chinese population is not well known. One study investigated the trend in prevalence of UC and CD in Wuhan city, central China, and evaluated the clinical features, extraintestinal manifestations, and the treatment of IBD in the last 14 years. Three hundred and eighty-nine patients with UC and 63 with CD were retrospectively collected from five central hospitals in Wuhan city, in which high-quality endoscopic and histological diagnoses were available from 1990 to 2003. UC and CD were diagnosed based on clinical, laboratory, radiological, endoscopic and histological examinations according to the internationally accepted Lennard-Jones criteria. The trend toward prevalence of UC and CD increased between 1990 and 2003 in Wuhan city. There was no change in the sex and age distribution comparing the two periods of 1990-1996 and 1997-2003, both in UC and CD. However, the number of individuals with higher education and a professional occupation from 1997 to 2003 was significantly higher than that during the period from 1990 to 1996 in patients with UC ($P \leqslant 0.004$). The mean age of patients with CD was significantly younger than that of UC at the time of diagnosis ($P < 0.0001$). The ratio of male to female patients was 1.53:1 in UC and 2.32:1 in CD, respectively.

The mean duration of onset of the disease to diagnosis was 1.4 years in UC and 1.1 years in CD. The extra-intestinal manifestations of UC and CD were 5.7% and 19%, and complications of UC and CD were 6.4% and 50.8%, respectively. Only 3% of UC patients required surgery, whereas 27% of CD patients underwent surgical procedures ($P < 0.001$). The prevalence of IBD has increased in Wuhan city, central China, but is not as high as in Western countries. The disease in Wuhan city has often been associated with young adult professional males with a high level of education. The clinical presentation of UC was often mild and had few extra-intestinal manifestations[4-6].

GENE MARKERS OF ULCERATIVE COLITIS IN THE CHINESE

Genetic factors play a significant role in determining IBD susceptibility. Many genes play a vital role in the development of IBD, including *TNF-308A*, *CARD15 (NOD2)*, *MIF-173*, *N-acetyltransferase 2 (NAT2)*, *NKG2D (natural killer cell 2D)*, *STAT6 (signal transducer and activator of transcription 6)*, *CTLA-4 (cytotoxic T lymphocyte antigen-4)*, *MICA-MICB (major histocompatibility complex A and B)*, *HLA-DRB1*, *HLA class-Ⅱ*, *IL-18* (*interleukin-18*), *IL-4*, *MICA-A5*, *CD14*, *TLR4*, *Fas-670*, *p53* and *NF-κB*.

TNF-308A

Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine that plays an important role in mediating inflammation and has been implicated in the pathogenesis of IBD. The regulation of TNF expression is genetically determined. The TNF gene lies on the short arm of chromosome 6 (6p21), 250 kb from the center of human leucocyte antigen-B (HLA-B), between HLA-B and DR, and within HLA Ⅱ. Recent studies have evaluated the role of TNF promoter polymorphisms in IBD but data are inconsistent. To date, few studies have reported the association of TNF promoter polymorphisms with susceptibility to UC in the Chinese Han ethnic population. Trans-racial mapping in an ethnically distinct but homogenous population may help clarify these associations. There is an association between TNF promoter polymorphisms and the susceptibility to UC in the Chinese Han ethnic population by genotyping for 6 common TNF promoter polymorphisms[6-9].

In a large sample study, a strong association between UC patients and the TNF-308A polymorphism was found in Japanese subjects[7]. No conclusive data on this association in European patients exist, however. This may reflect that the associations of TNF promoter polymorphisms with susceptibility to UC do vary among ethnic groups. Some scientists have supposed that TNF polymorphism is increased in IBD patients, even more than NOD2, and plays a more important role in the Asian population. IBD in the Asian population has unique epidemiological and clinical characteristics. For example, UC has a higher morbidity than CD in Asia. In an eastern China hospital between 1994 and 2003, 379 patients were diagnosed to have IBD. Of 379 patients, 317 had UC (83.6%) and this study shows similar characteristics of IBD to that in the West. However, there are some differences with respect to low severity and less extra-intestinal manifestations[8]. The ethnic and geographic differences may give important clues to the etiology of IBD. In the final analysis, genetic background plays a key role. To date, few studies have reported the association of TNF promoter polymorphisms with susceptibility to UC in the Chinese Han ethnic population. A number of studies have reported a high population attributed risk percentage of TNF promoter single nucleotide polymorphisms (SNPs), which reflects the higher mutant-type frequency in UC.

The importance of TNFα and the TNF receptor gene polymorphisms in the etiopathogenesis of IBD has not been elucidated. DNA from peripheral blood samples was obtained from 124 patients with CD, 106 patients with UC, and 111 unrelated healthy controls. Two SNPs of the *TNFα* gene, TNF (-308 G/A and -238 G/A), an SNP of the TNF receptor superfamily member 1A gene, TNFRSF1A (also known as TNFR1), at codon 12 in exon 1 (CCA/CCG), and two SNPs of the 1B gene, TNFRSF1B (also known as TNFR2), (1466 A/G and 1493 C/T) were examined. There was a difference in the carrier frequency for haplotype AG (-308 A, -238 G) between UC patients and the controls ($P < 0.01$). There was also a significant difference in carrier frequency for haplotype AT (1466 A, 1493 T) of the *TNFRSF1B* gene between CD patients and the controls ($P < 0.01$), and in those who were poor responders to treatment, which consisted of nutritional therapy, medical therapy and surgical therapy ($P < 0.001$). The authors suggest that one of the genes responsible for UC may be the *TNF* gene, or an adjacent gene, and that *TNFRSF1B* gene polymorphisms contribute greatly to the increased onset risk of CD and to the disease behavior[7].

Numerous studies from Europe and North America have provided a wealth of information regarding the epidemiological and clinical characteristics of IBD in Caucasians. Previous studies in mainland China have been limited by small patient numbers or by lack of detailed information about clinical subgroups of the disease. Cao *et al*[8] have assessed the demographic and clinical characteristics of IBD in Chinese patients. In the Sir Run Shaw Hospital between 1994 and 2003, 379 patients were diagnosed as having IBD. Demographic and clinical data were collected and analyzed. Of 379 patients, 317 had UC (83.6%, 168 male, 149 female, a male:female ratio of 1.13:1, age range at diagnosis 14-79 years, mean age 44 years) and 62 had CD (16.4%, 39 male and 23 female, a male:female ratio of 1.70:1, age range at diagnosis 13-70 years, mean age 33 years). In UC, 11.4% of patients had proctitis, 25.2% had proctosigmoiditis, 18.6% had disease in the splenic flexure and 44.8% had extensive colitis. Nine patients with UC (2.8%) had arthritis, and three (0.9%) had iritis or conjunctivitis. Of the 62 CD patients, 16 (25.8%) had diseases restricted to the terminal ileum, 15 (24.2%) had colonic diseases,

20 (32.3%) had ileocolonic disease and 11 (17.7%) had disease involving the upper gastrointestinal tract. This study showed similar characteristics of IBD to that in the West, but there are some differences with respect to severity and extra-intestinal manifestations. The ethnic and geographic differences may give important clues to the etiology of IBD[8].

Cao *et al*[9] reported the association with TNF-308A polymorphisms in Chinese patients with UC, suggesting that *TNF* gene may be a susceptibility gene for UC. The production of TNFα is elevated in TNF-308A carriers, resulting in excessive inflammation and onset of UC. The clinical application is to apply this new genetic information in the clinical setting to allow more rational therapies, selecting effective therapies (e.g. anti-TNF antibody) for refractory patients with UC, based on the genetic background. Further studies will be required to determine the functional effects of TNF-308A. Hereafter, authors can study gene knock-out mice, estimate the expression of TNFα in mutant cells using Northern and Western blotting, and investigate TNFα secretion by mutant-type cells after stimulation with LPS, compared with wide-type. We can also carry out a cohort study on correlation of TNFα expression level with *TNF-308A* genes and efficacy of anti-TNF antibody in UC patients based on the genetic background, in order to ascertain whether TNF-308A is responsible for UC[9].

Recent studies have evaluated the role of TNF promoter polymorphisms in IBD, but the data are inconsistent. Trans-racial mapping in an ethnically distinct but homogenous population may help clarify these associations. Cao *et al*[9] investigated the association between TNF promoter polymorphisms and susceptibility to UC in the Chinese Han ethnic population. They studied 110 unrelated UC patients and 292 healthy controls from Zhejiang Province, China. Genotyping for six common TNF promoter polymorphisms (TNF -1031T/C, -863C/A, -857C/T, -380G/A, -308G/A, -238G/A) was carried out by polymerase chain reaction sequence-specific primers (PCR-SSP). TNF -857T was increased in patients but without statistical significance ($P = 0.06$). Haplotype analysis revealed six haplotypes including two (H5 and H3), which contained TNF-308A. Of note, the rare haplotype H3 has not previously been identified in Caucasian populations. Homozygosis for the haplotype H4 comprising the common alleles at each TNF promoter single-nucleotide polymorphism (SNP) was negatively associated with disease ($P < 0.05$). The association with TNF-308A polymorphisms in Chinese patients with UC was reported by Cao *et al*[9]. The functional study in Chinese Han ethnic population is now required.

Progressive venous stenosis mediated, in part, by inflammatory cytokines is a major cause of synthetic hemodialysis graft failure. A TNFα gene polymorphism (G to A, position -308) has been shown to increase plasma cytokine levels and severity of diseases with an underlying inflammatory component. The TNFα-308 G/A and the TNFβ NcO1 polymorphisms have both been described to be associated with survival in sepsis or septic shock of various origins. That the TNFB2/TNFB2 genotype of the TNF-β NcO1 polymorphism is significantly associated with an increased risk for development of severe sepsis in severely injured blunt trauma patients was recently reported[9]. Up to now, neither functional consequences associated with these polymorphisms in inflammation nor the relationship of the TNFα -308 G/A polymorphism to the development of severe sepsis and its significance compared with the TNFβ NcO1 polymorphism have been determined for trauma patients.

The TNFα-308 allele may be related to susceptibility to UC. The *TNFα-308* gene polymorphism is not involved in pathogenesis of CD. No correlation was found between the TNFβ+252 polymorphism and IBD. Polymorphisms of the TNFα-308 and TNFβ+252 loci do not correlate with age, gender, disease activity or lesion site[8-10]. PCR and restriction fragment length polymorphism (RFLP) techniques were used to analyze gene polymorphisms in the *TNFα* and *TNFβ* genes in 131 patients with IBD[10]. The genotype frequency and allelic frequency of TNFα-308 in patients with UC were 15.5% and 8.7%, respectively, significantly higher than the control population ($P < 0.001$). There was no significant difference between patients with CD and the normal population with regard to the genotype frequency and allelic frequency of TNFα-308, and neither were there any differences with regard to TNFβ+252 in patients with IBD (UC and CD) and the normal population. The TNFα-308 polymorphism and the TNFβ+252 loci did not correlate with age, gender, disease activity or lesion site for IBD patients.

CARD15 (NOD2)

A lot of research has been undertaken on the genetic susceptibility of IBD. Genome-wide linkage studies focused on more than 10 chromosomal regions and fine-mapping of these regions have identified a number of genes, including *CARD15 (NOD2)*, *DLG5*, *OCTN1* and *2*, *TLR4* and *CARD4 (NOD1)*. With the recent completion of the human genome project, whole genome association studies (WGAS) have become possible and additional genes (*IL23R, IRGM, PTGER4, ATG16L1*) for CD and UC have been identified. At present, the *CARD15* gene is still the best understood susceptibility gene, explaining around 20% of the genetic predisposition to CD. Prediction of disease phenotype and response to the main therapies has for many years been a goal for physicians treating IBD patients. We now can accumulate some evidence, proving that genetic factors indeed influence both the clinical course of IBD patients and their likelihood of responding to certain therapies. Henckaerts *et al*[11] expected an exponential increase in the efforts devoted to research in this area. The optimal prediction of both disease behavior and response to therapy might result from combinations of clinical, biochemical, serological and genetic factors.

An insertion mutation at nucleotide 3020 (3020insC) in the caspase recruitment domain gene (*CARD15*),

originally reported as NOD2, is strongly associated with CD. The C-insertion mutation at nucleotide 3020 (3020inC) in the leucine-rich repeat (LRR) region results in a frameshift in the tenth LRR followed by a premature stop codon. This truncation mutation is responsible for the inability to activate nuclear factor (NF)-κB in response to bacterial lipopolysaccharide (LPS). The authors aimed to genotype *NOD2/CARD15* gene 3020insC frameshift mutation in Chinese patients with IBD. Guo *et al*[12] genotyped an insertion polymorphism affecting the leucine-rich region of the protein product by the allele-specific PCR in 74 unrelated patients with UC of Han nationality in Hubei Province of China, 15 patients with CD, and 172 healthy individuals. No significant differences were found in the genotype and allele frequencies of the C-insertion mutation of *NOD2* gene among patients with CD and UC and healthy controls. *NOD2* gene 3020insC frame-shift mutation is not a major contributor to the susceptibility to both UC and CD in Chinese Han patients[12].

The SNPs distribution of NOD2/CARD15 (R702W, G908R), OCTN1 1672C/T and OCTN2-207G/C in Chinese patients with IBD was investigated[13]. A total of 151 patients with UC, 61 patients with CD and 200 unrelated healthy controls were genotyped. Genotyping was performed by PCR-SSP or by RFLP analysis. Among the subjects in their study groups, including patients with CD, UC and healthy controls, none had OCTN and CARD15 variants, and very rarely, an IBD family history was found in their patients with the percentage of 0 (0/61 CD) and 1.3% (2/151 UC). The results indicate that although OCTN or CARD15 variation is associated with susceptibility to IBD in Western populations, these might be rare and may not be associated with susceptibility to IBD in Chinese patients[13].

The common variants in NOD2/CARD15 found in Caucasians with CD are not associated with CD in the Chinese Han population[14]. The three previously described SNPs associated with the development of CD in Caucasians are not found in Chinese patients with CD. None of the patients with CD had heterozygous or homozygous SNP variants. Similarly, none of the UC or dyspeptic controls had these SNPs[15].

Nucleotide oligomerization domain (NOD2) and human leukocyte antigen (*HLA*) genes are the most extensively studied genetic regions (IBD1 and IBD3 respectively) in IBD. Mutations of the *NOD2* gene are associated with CD and several *HLA* genes are associated with UC and CD. Toll like receptors (TLRs) play an important role in the innate immune response against infections by mediating recognition of pathogen-associated microbial patterns. Studying SNPs in molecules involved in bacterial recognition seems to be essential to define genetic backgrounds at risk of IBD. Recently, numerous new genes have been identified to be involved in the genetic susceptibility to IBD, such as NOD1/caspase-activation recruitment domains 4 (CARD4), chemokine ligand 20 (CCL20), IL-11, and IL-18. The characterization of these novel genes will lead to the identification of therapeutic agents and clinical assessment of phenotype and prognosis in patients with IBD[16].

MIF-173 gene

The etiology and pathogenesis of IBD is still unclear, but it has become evident that immune and genetic factors are involved in the process of IBD. Some cytokine gene polymorphisms such as TNFα, IL-1β and IL-1RA are known to be commonly associated with IBD. Macrophage migration inhibitory factor (MIF) is an important pro-inflammatory cytokine and plays a critical role in immune and inflammatory responses. MIF is implicated in a large number of immune and inflammatory diseases, including asthma, chronic hepatitis B, allergic neuritis and rheumatoid arthritis. Plasma MIF was reported elevated in patients with UC or CD compared with healthy controls. Anti-MIF antibodies reduced the severity of experimental colitis and limited the up-regulation of Th1-type cytokines. Anti-MIF antibodies are therefore of a potential therapeutic use in IBD. In the T lymphoblast cell line, the reverse situation was found with the MIF-173*C, significantly increasing the MIF expression under basal conditions. These differences in expression are likely to be due to differences in transcription factor interaction with the MIF-173 element. AP-4 transcription factor is a particular candidate[1,17] based on the promoter sequence analysis.

MIF-173 SNP was genotyped by tetra-primer amplification refractory mutation system (ARMS) and RFLP-PCR was also performed in 142 healthy subjects and 98 patients with IBD[17]. There were no discrepancies between the results obtained by tetra-primer ARMS and RFLP-PCR. The frequency of MIF-173 CC genotype was significantly higher in patients with UC (15.5%) than in healthy individuals (5.6%, $P = 0.018$). There was a trend towards a higher frequency of CC genotype among CD patients compared with healthy controls, however, this did not attain statistical significance ($P = 0.245$). MIF-173 CC genotype may be associated with susceptibility to UC. The results showed that the frequency of MIF-173 CC genotype was significantly higher in patients with UC than in healthy individuals. It suggested that MIF-173 CC genotype could be associated with susceptibility to UC. However CC genotype was not related to clinical features in patients with UC in Chinese Han population[17].

NAT2

Polymorphisms of NAT2 (N-acetyltransferase 2) acetylation may influence drug toxicity and efficacy and are associated with differential susceptibility to cancer. Acetylation phenotype may have clinical implications. NAT 2 is an enzyme that catalyzes the acetylation of harmful arylamines and has been implicated in various types of cancer. NAT 2 is primarily expressed in the hepatic system and intestinal epithelium, and is encoded at two polymorphic loci that give the phenotypic characteristics of slow and fast acetylation[18].

Arylamine NATs are xenobiotic-metabolizing enzymes responsible for the acetylation of many aromatic arylamine and heterocyclic amines, thereby playing an important role in both detoxification and activation of numerous drugs and carcinogens. Two closely related isoforms (NAT1 and NAT2) have been described in humans. NAT2 is mainly expressed in liver and gut, whereas NAT1 is found in a wide range of tissues. Inter-individual variations in NAT genes have been shown to be a potential source of pharmacological and/or pathological susceptibility. In addition, there is evidence that non-genetic factors, such as substrate-dependent inhibition, drug interactions or cellular redox conditions may also contribute to NAT activity. The recent findings provided possible mechanisms by which these environmental determinants may affect NAT activity. Interestingly, these data could contribute to the development of selective NAT inhibitors for the treatment of cancer and microbial diseases[18].

The wild type allele (NAT2*4) and three variant alleles (NAT2*5B, NAT2*6A and NAT*7B) of the *NAT2* gene were determined in 101 patients with IBD (84 patients with UC and 17 patients with CD) and 109 healthy controls by the RFLP-PCR method. Sixty-eight patients with IBD treated with SASP were followed up, and their adverse reactions were recorded. Eleven patients (16%) experienced adverse effects from SASP, including nine cases of sulfapyridine (SP) dose-related adverse effects and two cases of hypersensitivity (skin rash). Patients with the slow acetylator genotypes without the NAT2*4 allele experienced adverse effects more frequently (36%) than those with the fast acetylator genotypes with at least one NAT2*4 allele (11%), but the results were not significantly different ($P = 0.051$). However, those with the slow acetylator genotypes experienced more SP dose-related adverse effects than those with the fast acetylator genotypes ($P = 0.019$). The NAT2 gene polymorphism was not associated with susceptibility to IBD in Chinese populations, but the NAT2 slow acetylator genotypes were significantly associated with SP dose-related adverse effects of SASP in the treatment of IBD[19].

NKG2D

NKG2D (natural killer cell 2D) is an important activated cytokine that has been implicated in inflammatory reactions and the immune response. One of the best characterized NK cell receptors is NKG2D, a highly conserved C-type, lectin-like membrane glycoprotein expressed on essentially all NK cells, as well as on γδ-TcR+ T cells and αβ-TcR+ CD8+ T cells, in humans and mice. Recent studies implicating NKG2D in T cell and NK cell-mediated immunity to viruses and tumors, and its potential role in autoimmune diseases and allergenic bone marrow transplantation have been reviewed. NKG2D is a major activation receptor that associates with novel activation motif with DAP10 or ITAM containing KARAP/DAP12 adaptor molecules. A fundamental question is whether NK cell activation initiated *via* the H60-NKG2D interaction overrides the negative inhibition generated by the engagement of MHC class I to Ly49 receptors. Although an altered balance in the signaling strength of activating and inhibiting pathways of NK cells has been previously postulated. Recent findings illustrate that NK cell activation *via* NKG2D receptor can occur despite the normal expression of MHC class I molecules on the target cells[10-12]. By varying the levels of H60 expression and introducing additional MHC class I molecules on the target cells, Cao *et al*[20] demonstrated that the inhibitory Ly49 receptors can down-regulate NKG2D-mediated NK cell functions.

The function and the location of NKG2D gene show that it is an ideal susceptibility gene for UC. Cao *et al*[20] evaluated the NKG2D gene polymorphisms in patients from Zhejiang Province to determine whether the gene is associated with susceptibility to UC in the Chinese Han population. Blood samples were obtained from 110 patients with UC and 292 healthy controls in Zhejiang. Genotyping for two common NKG2D (10676G/, 908A/) polymorphisms was carried out using polymerase chain sequences with specific primers. NKG2D was not associated with disease (908A allele frequency 19.1% in patients *vs* 16.3% in controls, $P > 0.05$). Neither the patients with UC nor healthy controls had 10676G heterozygous or homozygous variants. The common variants in NKG2D are not associated with UC in the Chinese Han population. Research of larger samples and analysis from different layers and DNA sequences will help determine the function of NKG2D in the process of UC[20].

STAT6

STAT6 (signal transducer and activator of transcription 6) is a human gene. The protein encoded by this gene is a member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein plays a central role in exerting IL-4-mediated biological responses. IL-4 and IL-13 share many biological activities. To some extent, this is because they both signal *via* a shared receptor, IL-4Rα. Ligation of IL-4Rα results in activation of STAT6 and insulin receptor substrate (IRS) molecules. In T and B cells, IL-4Rα signaling contributes to cell-mediated and humoral aspects of allergic inflammation. It has recently become clear that IL-4 and IL-13 produced in inflamed tissues activate signaling in normally resident cells of the airway.

The *STAT6* gene is located on chromosome 12q13.3-14.1, just within the IBD2 region and is a key transcription factor involved in IL-4- and IL-13-mediated Th2 response. The G2964A polymorphism in the 3' untranslated region of the *STAT6* gene was studied in 84 unrelated Chinese patients with UC and 176 healthy controls by PCR and the amplification created restriction site method. The results were then compared with those from a Dutch study published previously. Significant

differences in genotype and allele frequencies of the STAT6 G2964A polymorphism were not found between patients with UC and healthy controls. Subgroups of the patients with UC classified according to the age at onset, sex and location of disease did not differ significantly in the distribution of this polymorphism. However, the genotypes ($P < 0.0001$) and allele frequencies ($P < 0.0001$) were significantly different between the Chinese and Dutch populations. The STAT6 G2964A polymorphism is not involved in the genetic susceptibility of Chinese patients to UC[21].

CTLA-4

CTLA-4 (cytotoxic T-lymphocyte antigen 4) is a glycoprotein expressed in activated T cells. CTLA-4 is essentially a costimulatory receptor that controls activation of T cells. In contrast to CD28, CTLA-4 delivers negative signals to T cells. *CTLA-4* gene is located on chromosome 2q33 and three *CTLA-4* gene polymorphisms in exon 1 (adenine or guanine at position) and in promoter -318, and a microsatellite (AT)n marker at position 642 of the 3'-untranslated region of exon 3. Recently, several independent studies reported a reduced inhibitory function of CTLA-4 in individuals with certain CTLA-4 genotypes. CTLA-4 consists of four exons that encode leader peptide, ligand-binding domain, transmembrane domain, and cytoplasmic tail, respectively. In humans, there are two isoforms of CTLA-4, which are a full-length isoform (flCTLA-4 transcript) and a soluble isoform (sCTLA-4 transcript) which lacks exon 3 by alternative splicing. Especially, sCTLA-4 is secreted and circulating in human sera. CTLA-4 is a negative regulator of T-cell proliferation and activation, which plays a critical role in the induction of self-tolerance and mediates antigen-specific apoptosis. Type 1 diabetes is a T-cell mediated autoimmune disease, therefore, its onset is partly associated with deficient expression and function of CTLA-4. Recent findings suggest that programmed cell death may also be involved in the pathogenesis of type 2 diabetes. Furthermore, there is evidence favoring a convergence in signaling pathways toward common effectors of beta-cell apoptosis elicited by stimuli implicated in the pathogenesis of type 1 and type 2 diabetes. If CTLA-4 were involved in this process, its association with type 2 diabetes might be conceivable. A functional role of the CTLA-4 A/G polymorphism, encoding a threonine to alanine change within the signal peptide of CTLA-4, has several possible explanations. It may be in linkage disequilibrium with the (AT)n microsatellite in the 3'-untranslated region and could, therefore, affect RNA stability. Equally, it may be in linkage disequilibrium with other disease-causing mutations[22-23]. However, it is also possible that this signal peptide polymorphism determines a subtle alteration in the subcellular localization of mature CTL A-4 protein, or affects the interaction of the nascent peptide with chaperonins, leading to a functionally important difference in the folding of the mature protein.

Eighty-seven patients with UC and 116 healthy controls were genotyped for CTLA-4 promoter -1722 and -1661 polymorphisms by RFLP-PCR in Hubei Province of central China. The frequency of "A/G + G/G" genotype at the -1661 site was significantly higher in UC patients than in healthy controls ($P = 0.002$). The frequency of the G allele at the -1661 site was also significantly higher in UC patients than in the controls ($P = 0.002$). However, the distribution of the genotypes at the -1722 site was not significantly different between the UC patients and the controls. The G allele of CTLA-4 promoter -1661 polymorphism showed a highly significant association with UC in the Han Chinese of central China[22]. One hundred unrelated Chinese patients with UC and 140 healthy controls were studied. The (AT) repeats in the 3' untranslated region of exon 4 of the CTLA-4 gene were amplified by allele-specific PCR. The amplified products were electrophoresed on a 120 g/L polyacrylamide gel, followed by silver staining. Twenty alleles were found in Chinese patients and healthy controls. The 122-bp allele was increased in UC compared with healthy controls ($P = 0.0001$). The frequency of the longer alleles ($\geqslant$ 118 bp) of UC was higher than that in healthy controls ($P = 0.0001$), but was not associated with location and severity of the disease. Furthermore, the longer alleles were not associated with haplotypes of C-318T/A+49G of the *CTLA-4* gene in Chinese patients with UC. The longer alleles of the *CTLA-4* gene microsatellite polymorphism were strongly associated with UC in Chinese patients[23].

Hou *et al*[24] studied 82 unrelated patients with UC and 204 healthy controls in a Chinese Han population. The frequency of the haplotype 2, 3 (-318C+49G/-318T+49A) was 26% in patients with UC and 41% in healthy controls ($P = 0.0147$), but this significance disappeared when Bonferoni correction was applied. No other significant differences in the distribution of allele and genotype frequencies were observed between *C-318T* and *A+49G* gene polymorphisms in UC of the Chinese Han population. The C-318T and A+49G polymorphisms of the *CTLA-4* gene were not associated with UC in Chinese Han patients[24].

CTLA-4 expressed mainly on activated T cells, inhibits T cell activation by combining B7 through competing CD28 and maintains immune system homeostasis. Polymorphisms in the *CTLA-4* gene are known to be associated with several autoimmune diseases, but no studies have related them to IBD. Sixty-eight unrelated Chinese Han patients with IBD (54 UC and 14 CD) and 140 healthy controls were studied. The (AT)n repeat sequence in the 3' untranslated region of exon 4 was amplified by allele-specific PCR. The amplified products were electrophoresed by 120 g/L polyacrylamide gel, followed by silver staining. Eighteen alleles of CTLA-4 microsatellite were found in Chinese patients and healthy individuals. A long allele of 122 bp was apparently increased in patients with UC compared with healthy controls ($P = 0.0002$). *CTLA-4* gene microsatellite polymorphism was strongly associated with UC in Chinese Han patients in Hubei Province[25].

Xia *et al*[26] studied 139 unrelated patients with UC, 163 patients with CD and 174 healthy controls of Dutch

Caucasian origin, as well as 35 patients with UC and 62 healthy controls from the Chinese Han population. No significant differences in the distribution of allele, genotype and haplotype frequencies were observed between *C-318T* and *A+49G* gene polymorphisms and IBD in Dutch Caucasians and UC in the Chinese Han population. Although the haplotypes of the C-318T and A+49G polymorphisms were distributed differently between Dutch Caucasian and Chinese Han populations, there were no differences in the subgroups of patients with CD classified according to age, localization and behavior in the Vienna classification and in those with UC classified according to age at onset, disease extension and presence of colectomy in the Dutch patients. However, the CTLA4-318 genotype CC was more frequent in patients with CD over 40 years of age (93%) than in younger patients (74%, $P = 0.045$). *C-318T* and *A+49G CTLA4* gene polymorphisms and their haplotypes are not associated with IBD in Dutch Caucasian patients and with UC in Chinese patients[26]. CTLA-4 polymorphism is not associated with UC in the Iranian population[27].

MICA-MICB

The 6D4 monoclonal antibody reacts with the human major histocompatibility complex (MHC) class I -related molecules A and B (MICA and MICB). MICA and MICB are related proteins of 83% amino acid similarity, and show homology with classical human leukocyte antigen (HLA) molecules. The structure of MICA and MICB is similar to classical HLA class I chains, however they do not bind β2 microglobulin or peptide typical of HLA class I. MICA and MICB are expressed on the cell surface of endothelial cells, fibroblasts, gastric epithelium and PHA-stimulated T cells, and act as a ligand for NKGD2 expressed on the surface of NK cells, γδ T cells and αβ CD8+ T cells. There is evidence suggesting that human cytomegalovirus subverts NK cell detection by inhibiting the function of MICB. Furthermore, MICA and MICB expression has been detected in several epithelial tumors isolated from breast, lung, ovary, prostate, colon and kidney. The MIC genes, which were described independently by two groups of investigators in 1994, encode proteins that are remotely similar to the HLA class I gene products. However, the MIC proteins do not associate with β2-microglobulin and have a groove that is too narrow to accommodate peptides for presentation to T cells.

MICA and MICB are stress-inducible cell surface antigens recognized by immunocytes bearing the receptor NKG2D, including intestinal epithelial Vδ1 γ/δ T cells, which may play a role in immunological reaction in intestinal mucosa. Lu *et al*[28] investigated the association of the microsatellite polymorphisms in the intron 1 of MICB and the MICA-MICB haplotype with the susceptibility to UC in the Chinese population. The microsatellite polymorphisms of MICB were genotyped in unrelated 127 Chinese patients with UC and 193 ethnically matched healthy controls by a semiautomatic fluorescently labeled PCR method. All the subjects were of Chinese Han ethnicity. The frequency of MICB-CA18 was significantly higher in UC patients compared with the healthy controls ($P = 0.0016$) and was increased in the female patients compared with the female healthy controls ($P = 0.0006$). Thus, MICB-CA18 is positively associated with UC patients in the Chinese population[28].

HLA class Ⅱ gene and HLA-DRB1 gene

The HLA region located on chromosome 6p encodes the highly polymorphic, classical class I and Ⅱ genes essential for normal lymphocyte function; it also encodes a further 224 genes. Many early studies investigating this region were limited by small sample size, poor statistical methodology, population stratification and variable disease definition. Although more recent studies have improved study design, investigators are still challenged by the complex patterns of linkage disequilibrium across this gene-dense region, and by the disease heterogeneity characteristic of all genetically complex disorders. Evidence is accumulating that both genetic and environmental factors contribute to UC. The most consistent genetic associations have been shown for the MHC locus HLA class Ⅱ alleles, but the IL-1 families of genes and the multidrug resistance gene MDR1 have also been implicated as genetic susceptibility factors for the development of disease. There is a relationship between UC and bacterial flora, with an increased number of adherent *Bacteroides spp.* and Enterobacteriaceae present in inflamed bowel segments[29].

DRB1 genotype is now thought to act mostly on disease phenotype. The presence of a double dose of RA-associated genes is associated with severe disease with cartilage destruction and increased frequency of extra-articular manifestations. IL-1 is the dominant cartilage-destructive cytokine and its impact on cartilage destruction can be reduced by regulatory cytokines such as IL-4 and IL-10. Increased frequency of particular polymorphism of *IL-1* and *IL-10* genes has been recently identified in the simultaneous presence of susceptible *DRB1* genes, and a specific polymorphism of exon 5 of the *IL-1β* gene is suggested to be predictive of erosive arthritis. Thus, the influence of DRB1 genotype on RA phenotype could be related to genetically controlled patterns of production of cytokines involved in cartilage erosion.

The pathogenesis of UC and CD is still unknown, but the importance of genetic susceptibility has been clearly shown by epidemiological data from family studies. Linkage studies have identified two susceptibility loci for IBD on chromosomes 12 and 16. Importantly, these linkages have been replicated by independent investigators, and studies of positional candidates within these regions continue, together with fine mapping strategies. Regions of suggestive linkage on chromosomes 1, 3, 4, 6, 7, 10, 22 and X have also been reported in individual studies. Other important candidate genes investigated include the IL-1 receptor antagonist, MUC3 and genes of the HLA system. The apparently conflicting data in different studies from around the world may be explained by ethnic differences,

case mix and genetic heterogeneity. Replicated class Ⅱ HLA associations include HLA DRB1*0103 and DR2 (DRB1*1502) involved in UC susceptibility, and HLA DRB1*03 and DR4 as resistance alleles for CD and UC, respectively. Animal studies have provided insights from targeted mutations and quantitative trait locus analysis. The goals of continuing research include narrowing the regions of linkages and analysis of candidate genes, and the application of newly developed methods using SNPs. Advances in IBD genetics hold the potential to provide knowledge about the disease pathogenesis at the molecular level, with ensuing benefits for clinical practice[29-30].

Antigen presentation by MHC class Ⅱ molecules plays an important role in controlling immunity and autoimmunity. Multiple co-factors including the invariant chain (Ii), HLA-DM and HLA-DO are involved in this process. Chen *et al*[30] found that DO inhibits presentation of endogenous self-antigens and that development-regulated DO expression enables antigen-presenting cells to preferentially present different sources of peptide antigens at different stages of development. Disruption of this regulatory mechanism can result in not only immunodeficiency but also autoimmunity. Clinical tests for any of these potential genetic defects are not yet available. They proposed the use of multi-color flow cytometry in conjunction with intracellular staining to detect expression of Ii, DM and DO in peripheral blood B cells, as a convenient reliable screening test to identify individuals with defects in antigen presentation.

Subgroups of UC patients have been further defined by the presence of anti-neutrophil cytoplasmic antibodies (ANCAs). Lee *et al*[31] attempted to define the *HLA class Ⅱ* genes (DRβ, DQα, DQβ) and their relationship with ANCA in southern Chinese patients with UC. Patients were tested for class Ⅱ genes by RFLP and PCR. The indirect immunofluorescence test was used to detect ANCA in the sera. Ethnically matched normal controls were used for comparison. In ANCA-positive UC patients, there was a strong association with the HLA-DQα1c allele ($P < 0.0001$) when compared with controls. This association was not found in ANCA-negative UC patients ($P = 0.21$). In Chinese UC patients, ANCA positivity is associated with the HLA-DQα1c allele, which is not the case in Caucasian patients[31].

Three human mucin cDNAs (Muc-1, Muc-2 and Muc-3) have recently been cloned and sequenced. The major portion of each mucin consists of sequences repeated in tandem along the protein. Three mucins are distinct due to differences in tandem repeat length, lack of sequence homology and different chromosomal locations of their genes. Since altered mucin glycosylation occurs in cancer, resulting in exposure of core carbohydrate, Yuan[32] postulated that increased exposure or other alteration of core peptide structure might occur in cancerous tissues. Antibodies against Muc-1, Muc-2 and Muc-3 tandem repeats were used for immunohistochemical analysis of normal, non-malignant and cancer tissues. The results indicate that, in normal tissues, only Muc-2 was expressed, while in cancerous tissues, all three mucin core peptides were significantly accumulated. All of the three mucin core peptides were increasingly expressed in adenoma, dysplastic epithelium and active UC (pre-malignant lesions), but not in hyperplastic polyps, ischemic colitis and quiescent UC (non-malignant diseases)[32].

The genetic factors predisposing to UC have remained totally unclear to date. HLA-DRB1 genotyping was carried out in 72 unrelated patients with UC and 314 healthy controls using PCR-SSP[33]. All of the patients and healthy controls are Han people in China. The frequency of DRB1*07 allele was increased in UC patients compared with healthy controls ($P = 0.0229$), but the significance disappeared when Bonferroni correction was applied ($P = 0.2977$). Furthermore, compared with healthy controls, although HLA-DRB1*07, DRB1*16/DRB1*09 and DRB1*07/DRB1*12 genotypes were increased in frequency in the patients with extensive colitis, and the patients without extra-intestinal manifestations (EIMs) carried an increased frequency of HLA-DRB1*07 and DRB1*07/DRB1*12 genotypes, although these differences did not reach statistical significance after Bonferroni correction. HLA-DRB1 alleles showed no strong association with UC, and no HLA-DRB1 alleles or genotypes were strongly associated with clinical subgroups of UC in Chinese patients[33].

IL-18 and IL-4 genes

IL-18 is a pro-inflammatory cytokine. Although IL-18 has been implicated as a mediator of antibacterial defense, detrimental effects of IL-18 during bacterial infections have also been demonstrated. Microglia and astrocytes can produce IL-18. *Streptococcus. pneumoniae* is an important microorganism in meningitis. IL-18 plays an important role in sarcoidosis by inducing IFN-γ. The roles of -137 (G/C), -607 (C/A), and -656 (G/T) SNPs of *IL-18* gene promoter regions were compared between 176 individuals in a control group and 161 patients in an experimental group. The major haplotypes-137G/-607C/-656G had a higher promoter activity under the stimulus of sodium butyrate than another major haplotype, -137G/-607A/-656T. This coincided with the genotype with a high IL-18 concentration in the serum. Smokers had a significantly shorter clinical course than non-smokers. A difference in protein expression based on the disparities of *IL-18* gene promoter activity explains the different clinical picture for sarcoidosis, and suggests the effect of smoking on the disease[34].

IL18 was mapped to 11q22.2-22.3 in 1998. Owing to IL-18's important and novel role in immunomodulation, the gene itself has been subject to scrutiny, with the aim of discovering variants that may affect disease susceptibility and/or progression. Despite being sequenced numerous times in different populations, no non-synonymous variants have been found. However, a number of polymorphisms within the proximal promoter have been verified that may interfere with transcription-factor-binding sites. Many of the subsequent association analyses have centered on these

variants, but have yielded no consistent results, despite numerous different study populations being genotyped. IL18 has recently been resequenced in its entirety, enabling the tagging SNP methodology to be adopted. This approach has yielded interesting results, with genetic variation affecting protein levels, and risk. The review by Thompson *et al*[34] aims to compile and reflect the data of interest published to date, with a focus on the diseases related to aberrant inflammatory control.

Under normal situations, the intestinal mucosa is in a state of 'controlled' inflammation regulated by a delicate balance of proinflammatory (TNFα, IFNγ, IL-1, IL-6, IL-12) and anti-inflammatory cytokines (IL-4, IL-10, IL-11). The mucosal immune system is the central effector of intestinal inflammation and injury, with cytokines playing a central role in modulating inflammation. Cytokines may, therefore, be a logical target for IBD therapy using specific cytokine inhibitors. Biotechnology agents targeted against TNF, leukocyte adhesion, Th1 cell polarization, T-cell activation or NF-κB, and other miscellaneous therapies are being evaluated as potential therapies for IBD. In this context, infliximab is currently the only biological agent approved for the treatment of inflammatory and fistulizing CD. Other anti-TNF biological agents have emerged, including CDP 571, certolizumab pegol (CDP 870), etanercept, onercept and adalimumab. However, ongoing research continues to generate new biological agents targeted at specific pathogenic mechanisms involved in the inflammatory process. Lymphocyte-endothelial interactions mediated by adhesion molecules are important in leukocyte migration and recruitment to sites of inflammation, and selective blockade of these adhesion molecules is a novel and promising strategy to treat UC and CD. Therapeutic agents that inhibit leukocyte trafficking include natalizumab, MLN-02 and alicaforsen (ISIS 2302). More controlled clinical trials are currently being conducted, exploring the safety and efficacy of old and new biological agents and the research certainly will open new and exciting perspectives on the development of therapies for IBD.

Eighty-one UC patients and 114 healthy subjects were enrolled by Peng *et al*[35]. *IL-1β*, *IL-1RA* and *IL-4* gene polymorphisms were analyzed with RFLP-PCR and PCR-SSP, respectively. The gene frequency of allele RP2 of IL-4 in patients with UC was significantly higher than that in healthy subjects ($P = 0.00002$), but the gene frequency of allele RP1 in HS was significantly higher than that in UC patients ($P = 0.00002$). The OR of the genotype RP1.2 and RP2.2 was 2.71 and 9.04 respectively. There was no difference in the gene frequencies of IL-1β and IL-1RA between patients with UC and healthy subjects ($P > 0.05$). When patients with UC were divided into ANCA-positive and -negative groups, there was a significant difference in the gene frequencies of allele RP1 and RP2 of IL-4 between the two groups ($P < 0.05$). There is a correlation between the Chinese UC patients and the gene polymorphisms of intron 3 of IL-4. The gene frequency of allele RP1 in UC patients is lower, but the gene frequency of allele RP2 is significantly higher. The differences in gene frequencies of IL-4 between the UC patients and healthy subjects are mainly found in the ANCA-positive UC patients. The Chinese UC patients are not associated with IL-1β and IL-1RA gene polymorphisms[35].

MICA-A5

The role of MICA protein in the immune response is unknown. Recently, it was shown that this polymorphic molecule is mainly expressed by epithelial cells and interacts with the γδT cells. γδT cells appear to dominate lymphocyte populations isolated from epithelium. T lymphocytes bearing γδ receptors have also been isolated from the female genital tract. Expression of MICA by cervical epithelium and its recognition by γδT cells suggest that it may be important in immune surveillance and direct induction of mucosal immunity[35-37]. IBD arises in part from a genetic predisposition, through the inheritance of a number of contributory genetic polymorphisms. These variant forms of genes may be associated with an abnormal response to normal luminal bacteria. A consistent observation across most populations is that any of three polymorphisms of the caspase-activated recruitment domain (CARD15) gene are more prevalent in IBD patients as compared with unaffected controls. Similar aberrant responses to bacteria are associated with variants in autophagy-related 16-like 1 (ATG16L1) and human defensin (HBD-2, -3 and -4) genes. The defective bacterial signal in turn leads to an excessive immune response, presenting as chronic gut inflammation in susceptible individuals. Inconsistent population reports implicate the MHC, which encodes a number of HLA, antigens MICA or cytokines, such as TNFα. Toll-like receptors encoded by the *TLR4* or *TLR9* genes may also play a role. Recent whole genome scans suggest that a rare variant in the IL-23 receptor (IL23R) gene may actually protect against IBD. Other implicated genes may affect mucosal cell polarity (Drosophila discs large homologue5, DLG5) or mucosal transporter function (sodium dependent organic transporters, SLC22A4 and SLC22A5). A variant in ABCB1 (ATP-binding cassette subfamily B member 1) may be especially associated with increased risk of UC. While pharmacogenetics is increasingly being used to predict and optimize clinical response to therapy, nutrigenetics may have even greater potential. In many cases, IBD can be controlled through prescribing an elemental diet, which appears to act through modulating cytokine response and changing the gut microbiota. More generally, no single group of dietary items is beneficial or detrimental to all patients, and elimination diets have been used to individualize dietary requirements. However, recognizing the nature of the genes involved may suggest a more strategic approach. Pro- or prebiotics will directly influence the microbial flora, while immunonutrition, including omega-3 fatty acids and certain polyphenols, may reduce the symptoms of gut inflammation. The expression of gut transporters may be modulated through various herbal remedies, including green tea polyphenols. Such approaches would

require that the gene of interest is functioning normally, other than its expression being up- or down-regulated. However, new approaches are being developed to overcome the effects of polymorphisms that affect the function of a gene. A combination of human correlation studies with experimental models could provide a rational strategy for optimizing nutrigenetic approaches to IBD[36].

MICA plays a role in regulating protective responses by intestinal epithelial Vδ1 γδT cells and the polymorphisms of MICA were reported to be related to several autoimmune diseases. Henckaerts *et al*[11] investigated the association of the microsatellite polymorphisms of TM region of the *MICA* gene with the susceptibility to UC in the Chinese population. The microsatellite polymorphisms of the MICA gene were genotyped in 86 unrelated Chinese patients with UC and 172 ethnically matched healthy controls by a semiautomatic fluorescently labelled PCR method. All the subjects were of Chinese Han ethnicity. The frequency of MICA-A5.1 homozygous genotype and A5.1 allele was significantly increased in UC patients compared with healthy controls ($P = 0.0009$ and $P = 0.0014$). When adjusting for the effects of gender and age at onset, MICA-A5.1 homozygous genotype and A5.1 allele were also increased in the UC patients. Moreover, MICA-A5.1 allele was significantly increased in frequency in the female UC patients ($P = 0.0095$). Logistic regression analysis also revealed that gender was independently associated with UC patients carrying the MICA-A5.1 allele ($P = 0.046$), although the UC patients with extensive colitis ($P = 0.005$) and those with EIMs ($P = 0.0039$) were more likely to carry the MICA-A5.1 allele. EIMs were associated with extent of disease ($P < 0.0001$) and MICA-A5.1 allele was not associated with UC patients with extensive colitis or with EIMs in the logistic regression analysis. Therefore, the MICA-A5.1 homozygous genotype and A5.1 allele were closely associated with UC and the MICA-A5.1 allele was positively associated with the female UC patients in the Chinese population[37].

CD14 and TLR4 genes

TLR and CD14 are components of the lipopolysaccharide receptor complex. A large volume of has been research undertaken on the genetic susceptibility of IBD. Genome-wide linkage studies pointed towards more than 10 chromosomal regions, and fine-mapping of these regions led to the identification of a number of genes, including *CARD15* (*NOD2*), *DLG5*, *OCTN1* and *2*, *TLR4* and *CARD4* (*NOD1*). With the recent completion of the human genome project, whole genome association studies have now become possible and have identified additional genes (*IL23R*, *IRGM*, *PTGER4*, *ATG16L1*) for CD and UC, which have subsequently been replicated. At present, the CARD15 gene is still the most understood susceptibility gene, explaining around 20% of the genetic predisposition to CD. Prediction of disease phenotype and response to the main therapies has for many years been a goal for physicians treating IBD patients. Only now, we have started to accumulate some evidence proving that genetic factors indeed influence both the clinical course of IBD patients and their likelihood of responding to certain therapies. In the coming years, we expect an exponential increase in the efforts devoted to research in this area. The optimal prediction of both disease behavior and response to therapy might result from complex combinations of clinical, biochemical, serological and genetic factors[38].

RFLP-PCR was used to genotype polymorphisms TLR4 Asp299Gly and CD14 C-260T in 114 patients with UC and 160 healthy controls in the Chinese Han population. Moreover, a comparison was made with 170 healthy Dutch white subjects[39]. No TLR4 Asp299Gly mutation was detected in any patients or healthy controls in the Chinese Han population, which was similar to Japanese subjects, but the mutation occurred in 10% of the Dutch white subjects. There were no significant differences of CD14 genotypes between healthy controls and the patients in Chinese patients with UC.

Fas-670 gene

Fas (Apo-1/CD95) antigen is a 45-kDa type I membrane protein, which is expressed in various tissues and cells. Fas is a member of the TNF superfamily and mediates apoptosis when cross-linked with agonistic anti-Fas antibody or Fas ligand (FasL). Although the best-characterized physiological system involving Fas/FasL-mediated apoptosis is observed in the immune system, a role of Fas/FasL in non-lymphiod tissues has become increasingly evident. Fas-mediated apoptosis is thought to be involved in autoimmune disease and inflammatory disorders. Recent studies have suggested that immune dysregulation and genetic factors play important roles in the pathogenesis of IBD. Defective apoptosis of lamina propria T cells may be a factor in mucosal immune dysregulation and tissue inflammation. One of these polymorphisms is a single nucleotide substitution at the -670 position that alters the *Mva* I restriction enzyme cutting site, creating an RFLP. This polymorphism is situated at the consensus sequence site, the gamma interferon activation site. This site can bind to transcription factors such as STATs, thus exerting an effect on the level of transcription of the Fas protein. Although expression and functional effects of the Fas antigen have been found to be associated with IBD, the relationship between Fas-670 polymorphism and IBD has not been reported yet. In a recent study, Peng *et al*[35] could not find any significant association between Fas-670 polymorphism and IBD, which indicates genetic heterogeneity of the diseases. Since Fas-670 polymorphism does not contribute to IBD, there may be other genes that are involved in the pathogenesis of IBD, and other mechanisms of gene regulation may influence Fas-mediated epithelial apoptosis in IBD.

For the Fas-670 polymorphism, it has been hypothesized that either increased apoptosis of intestinal epithelium or decreased apoptosis of lamina propria lymphocytes may induce inflammation of the gut. Fifty unrelated Chinese patients with IBD (38 patients with UC and 12 with CD) and 124 healthy controls were

genotyped for the Fas-670 polymorphism by RFLP-PCR. The PCR product was digested by *Mva* I restriction enzyme. Distribution of the Fas-670 gene polymorphism was 33% for the AA genotype, 52% for the AG genotype and 15% for the GG genotype in 124 healthy subjects, and 30% for the AA genotype, 42% for the AG genotype and 28% for the GG genotype in patients with IBD. However, there was no significant difference in the genotype (P = 0.1498), allele frequencies (P = 0.3198) and carriage frequencies (P = 0.4133) between healthy controls and IBD patients. Furthermore, no difference was found between left-sided and total colitis (P = 0.8242). Fas-670 polymorphism is not associated with IBD in Chinese patients. In a recent study, Xia *et al*[40] genotyped Fas-670 polymorphism in Chinese patients with IBD and healthy controls, and found that the polymorphism was not associated with UC and CD. The study suggested that Fas-670 polymorphism might not play a role in susceptibility of IBD in Chinese patients.

p53 gene

The *p53* gene like the *Rb* gene is a tumor suppressor gene, i.e. its activity stops the formation of tumors. If a person inherits only one functional copy of the *p53* gene from their parents, they are predisposed to cancer and usually develop several independent tumors in a variety of tissues in early adulthood. This condition is rare, and is known as Li-Fraumeni syndrome. However, mutations in p53 are found in most tumor types, and so contribute to the complex network of molecular events leading to tumor formation. The *p53* gene has been mapped to chromosome 17. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (Cdk2). When p21 is combined with Cdk2, the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the stop signal for cell division. Thus cells divide uncontrollably, and form tumors.

p53 protein expression was detected by immunohistochemistry in 70 specimens from 21 cases of UC and 25 colonic mucosa specimens from normal subjects. The specimens of UC were examined for the mutation in exon 5, 6, 7, 8 of *p53* gene with the microdissection-PCR-SSCP/HA-clone-sequencing technique and the alterations in 10 microsatellite loci with the microdissection-PCR-SSLP-clone-sequencing technique[41]. None of 25 normal specimens was p53-positive immunohistochemically, while 4/21 of UC specimens were p53-positive. p53-positive rate in inflammatory mucosa of UC specimens was 0/5, and 1/7, 2/7 and 1/2 in low-grade displasia (LGD), high-grade displasia (HGD) and carcinoma, respectively. The abnormal exons were detected by SSCP and confirmed by sequencing in two out of 21 cases: one was exon 6 in a case with carcinoma and the other was exon 8 in an HGD case; both had positive p53 expression. Two cases were positive in the Bat26 locus by SSLP: one was an LGD case, and the other was a case of carcinoma, which also had abnormal exon 6 of *p53* gene. Another nine microsatellite loci, [TGFβRII (A) (10), IGFIIR (G) (8), IGFIIR (CT) (5), TGFβRII (GT) (3), BAX (G) (8), hMSH3 (A) 8, hMSH6 (C) (8), TCF4 (A) (9) and DPC4 (CA) (17)] were negative in all cases. The *p53* gene mutations and microsatellite instability may be one of the mechanisms for higher risk of carcinogenesis in UC[42].

NF-κB

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that acts as a transcription factor. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection. Consistent with this role, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κB has also been implicated in the processes of synaptic plasticity and memory.

In the healthy gut, the mucosal immune system ensures the balance between pro- and anti-inflammatory mediators, thereby allowing an effective defense against luminal pathogens, but at the same time prevents an overwhelming immune reaction directed against the huge amount of harmless luminal antigens (e.g. components of food or non-pathogenic bacteria). In both entities of IBD (CD and UC), this immunological balance is severely impaired and shifted towards the pro-inflammatory side. The chronic mucosal inflammation in IBD is caused by hyperactivation of effectors immune cells, which produce high levels of pro-inflammatory cytokines like TNFα, IL-6 and IFNγ, resulting in colonic tissue damage. NF-κB was identified as one of the key regulators in this immunological setting. Its activation is markedly induced in IBD patients, and through its ability to promote the expression of various pro-inflammatory genes, it strongly influences the course of mucosal inflammation. Considering the different cell-type specific effects that are mediated by NF-κB, the authors described its complex role in IBD and discussed the existing pharmacological attempts to block the activation of NF-κB to develop new therapeutic strategies in IBD.

A total of 27 cases of UC were investigated. Fifteen cases received sulfasalazine (SASP) treatment or SASP and glucocorticoid treatment, and 12 patients did not receive any medication related to UC[41]. Normal mucosa from nine colon cancer patients served as a control. Ten pieces of intestinal mucosal biopsy specimens were obtained from each patient. The mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were determined by RT-PCR. The protein levels of ICAM-1 and VCAM-1 were measured by ELISA. NF-κB DNA binding activity was evaluated by electrophoretic mobility shift assay (EMSA). The results showed that NF-κB DNA binding activity, mRNA and protein expression of

ICAM-1 and VCAM-1 were increased significantly in patients with UC compared with normal control ($P < 0.05$). Glucocorticoids and SASP markedly inhibited NF-κB activation and significantly decreased mRNA and protein expression of ICAM-1 and VCAM-1 ($P < 0.05$). Adhesive molecule (ICAM-1 and VCAM-1) gene activation had significant positive correlation with the NF-κB DNA binding activity ($P < 0.05$, $P < 0.05$, respectively). NF-κB is a major and essential factor in regulating the expression of adhesive molecules; it plays an important role in the pathogenesis of UC. SASP and glucocorticoids ameliorate UC *via* inhibition of NF-κB activation and reduction of adhesive molecule expression[42].

Among the 31 patients with UC, 17 patients received SASP or SASP and glucocorticoid treatment, and 14 patients did not receive any medication related to UC. Normal mucosa from 11 colon cancer cases served as a control. Ten pieces of intestinal mucosal biopsy specimens were obtained from each patient. NF-κB DNA binding activity was evaluated with EMSA. Expression of cytokine mRNA was studied RT-PCR. The expression of IL-1β and IL-8 mRNA was increased significantly in patients with UC, as compared with that in the control specimens ($P < 0.05$), and had a significant positive correlation with NF-κB DNA binding activity ($P < 0.05$, $P < 0.05$, respectively). Glucocorticoids and SASP strongly inhibited NF-κB activation and significantly decreased the expression of IL-1β and IL-8 mRNA. NF-κB is a major and essential factor in regulating the expression of cytokine and plays a fundamental role in the pathogenesis of UC. SASP and glucocorticoids decreased cytokine expression *via* inhibition of NF-κB activation[43].Various components of the mucosal immune system are implicated in the immunopathogenesis of UC. Evidence from animal models also suggests that an altered immune response to the commensal microflora of the host plays a central role in the development of UC. Therefore, it is elucidated that the cells and molecules are implicated in the immunopathogenesis of the disease from four aspects: antigens in the intestine, dendritic cells, TLRs and NF-κB in UC[44].

Ten pieces of colon mucosal biopsy specimens were obtained from 31 patients with UC, 17 of whom received SASP or SASP plus glucocorticoid and 14 received no medication. Samples of normal mucosa around the lesion taken from 11 patients with colon cancer were used as controls. NF-κB DNA binding activity was evaluated by EMSA. NF-κB p65 expression was determined by Western blot analysis and immunohistochemical staining with a NF-κB p65 antibody. The type of cells containing activated NF-κB p65 was identified by double immunofluorescence confocal laser scanning microscopy. The expression of NF-κB p65 and NF-κB DNA binding activity was significantly higher in patients with UC than in the controls ($P < 0.05$), and was correlated with the degree of inflammation. The NF-κB expression was significantly stronger in the nuclei than in the cytoplasm in patients with UC without pharmacotherapy. The NF-κB expression in nuclei was significantly stronger in the group without pharmacotherapy than in the group with pharmacotherapy ($P < 0.05$). Only a few NF-κB p65-positive cells were seen in the controls. NF-κB p65 expression was found in all major subsets of mononuclear cells, including macrophages, B lymphocytes, T lymphocytes, and cryptal epithelial cells. The increased activation of NF-κB and increased expression of NF-κB may be involved in the pathogenesis of UC. Glucocorticoids and SASP strongly inhibited NF-κB activation and expression. The inhibition of NF-κB activation may be a central part of the anti-inflammatory action of glucocorticoids and SASP, which might represent an important pharmacological mechanism in the treatment of patients with UC. NF-κB will be an important target for cytokine-based therapy of UC[45].

Table 1 Epidemiology and gene markers of IBD[2-6,48,49]

Epidemiology	Gene markers of IBD
Incidence per 100 000	0.5-2.0 (China); 2-14 (North America)
Prevalence per 100 000	1-23 (China); 26-246 (North America)
Geography	Northern Countries > Southern Countries; Lower (China)
Age of onset (yr)	20-35 (China)
Sex	M > F (China)
Race	Whites > Blacks; Lower (Chinese)
Possible genetic associations (Chinese)	*TNF-308A*, *CARD15* (*NOD2*), *MIF-173*, NAT2, *NKG2D*, *STAT6*, *CTLA-4*, *MICA-MICB*, *HLA-DRB1*, *HLA class Ⅱ*, *IL-18*, *IL-4*, *MICA-A5*, *CD14*, *TLR4*, *Fas-670*, *p53*, *NF-κB*; Chromosome 3, 5, 7, 12, 16 ,19

CONCLUSION

Epidemiology and genetic research in IBD (UC and CD) has provided knowledge about the complexity and heterogeneity of the disease and has started to correlate the interactions between genetic and environmental risk factors in IBD; however, the complex genetic background that allows the development of IBD is not fully understood. Understanding the pathways in which genetic factors influence IBD will uncover pathogenesis of the disease, offer more accurate diagnosis, and ultimately lead to the development of better new drugs and therapies. The most important advance toward understanding this process has been identification of specific genetic associations with IBD, which will shed new light on future research of IBD. Researchers are studying how and why the immune system is activated, how it damages the colon, and the processes involved in healing. Currently, numerous clinical trials on UC are being conducted. Immunomodulators used for treating severe UC include azathioprine/6-MP, methotrexate and cyclosporine. Integrated traditional Chinese and modern medicine is safe and effective in maintaining remission in patients with UC[1,11,46]. There are also complementary and alternative therapies for IBD[47]. Epidemiology and gene markers of IBD are shown in Table 1.

UC and CD are complex polygenic disorders, characterized by several genes, together with environmental factors contributing to the development of IBD. Recent advances in research on genetic susceptibility have allowed the identification of diverse genes at different levels, innate immunity, antigen presentation molecules,epithelial integrity, drug transporters and cell adhesion. The application of genetic testing into clinical practice has become available and all genetic markers may have several clinical implications: prediction of disease phenotype, molecular classification, prevention of complications, and prognosis.

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TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Bile acids: Chemistry, physiology, and pathophysiology

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Abstract

The family of bile acids includes a group of molecular species of acidic steroids with very peculiar physical-chemical and biological characteristics. They are synthesized by the liver from cholesterol through several complementary pathways that are controlled by mechanisms involving fine-tuning by the levels of certain bile acid species. Although their best-known role is their participation in the digestion and absorption of fat, they also play an important role in several other physiological processes. Thus, genetic abnormalities accounting for alterations in their synthesis, biotransformation and/or transport may result in severe alterations, even leading to lethal situations for which the sole therapeutic option may be liver transplantation. Moreover, the increased levels of bile acids reached during cholestatic liver diseases are known to induce oxidative stress and apoptosis, resulting in damage to the liver parenchyma and, eventually, extrahepatic tissues. When this occurs during pregnancy, the outcome of gestation may be challenged. In contrast, the physical-chemical and biological properties of these compounds have been used as the bases for the development of drugs and as pharmaceutical tools for the delivery of active agents.

Key words: Cholestasis; Cholesterol; Liver; Metabolism; Transport

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INTRODUCTION

Over the last decades the interest of hepatologists in bile acids has grown markedly[1]. The reason has been the discovery of the role of these acidic steroids in many different physiological processes, which has important implications from the point of view of liver and intestinal pathology and pharmacology. Moreover, in recent years their use in supramolecular chemistry, materials chemistry and nanotechnology has been the focus of intensive research[2]. Bile acids include a group of molecular species with similar, but not identical, chemical structures. Surprisingly, they exhibit diverse physical properties and even more divergent biological characteristics. Although their best-known role is their participation in the digestion and absorption of fat, they play an important role in several other functions. In the present review, these roles will only be mentioned briefly because they are addressed in depth in other reviews of this series. The relevance of their physiological roles explains why genetic abnormalities accounting for alterations in their synthesis, biotransformation and/or transport may result in severe alterations, even leading to lethal situations, for which, in pediatric patients, the sole therapeutic option may be liver transplantation.

Moreover, the increased levels of bile acids that may

be reached during cholestatic liver diseases are known to induce oxidative stress and apoptosis that results in damage to the liver parenchyma and, eventually, extrahepatic tissues. When this occurs during gestation, such as in women suffering from intrahepatic cholestasis of pregnancy, the outcome of the gestational process and/or the health of the fetus may be challenged. These aspects will be also be considered in depth in a separate review of this series.

In contrast to the involvement of bile acids in the etiology and pathogenesis of several diseases, the physical-chemical and biological properties of these compounds have permitted them to be used in the development of drugs and as pharmaceutical tools for the delivery of active agents, as will be commented below.

PHYSICAL-CHEMICAL CHARACTERISTICS OF BILE ACIDS

Chemical structure

In the common biomedical literature, the terms "bile acids" or "bile salts" are generally used to denote the so-called "modern" bile acids[3]. They have 24 carbon atoms and are abbreviated as C_{24} bile acids, in contraposition to "primitive" bile acids, which have 25-27 carbon atoms (C_{27}, C_{26}, C_{25} bile acids) and are present in the bile acid pool of primitive (e.g. coelacanth and sharks) and less primitive (e.g. reptiles and amphibians) vertebrates. The structures of some of the most abundant bile acids in humans are depicted in Figure 1. In higher vertebrates, C_{24} bile acids constitute a major part of the bile[4], and in human bile, these compounds are almost completely in conjugated form with either glycine (75%) or taurine (25%)[5]. Under physiological conditions, conjugation increases their water-solubility.

Bile salts have a unique and fascinating molecular structure derived from a saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene system, usually known as the steroid nucleus. The steroid nucleus is also the main carbon skeleton of other families of compounds such as brassinosteroids, ubiquitously distributed throughout the plant kingdom[6], hopanoids, commonly used as biomarkers in organic geochemistry[7], triterpenoids[8], and hormones.

The steroid nucleus consists of three six-member rings (A, B and C) and a five-member ring (D), with a curved (beaked) or flat structure (depending on a *cis*- or *trans*-fused configuration between the A and B rings). In mammals, the nucleus is almost invariably 5β (A/B junction in *cis* configuration), while in lower vertebrates, some bile acids, known as *allo*-bile acids, exhibit an A/B *trans*-fusion. There are 11 chiral carbon atoms. Bile acid molecules are approximately 20 Å long, with an average radius of about 3.5 Å (Figure 2).

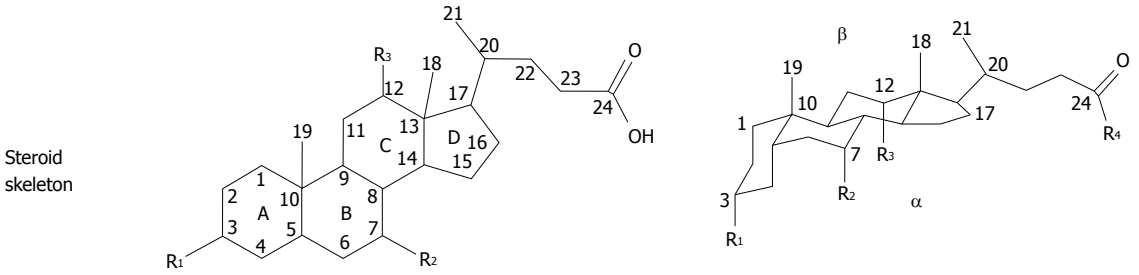
As early as the 1960s, Haslewood had noticed the biological significance of chemical differences in bile salts[9] and that the chemical nature of the bile salts of more primitive animals clearly indicates that an evolution from C_{27}, 5α-alcohol sulfates to C_{24}, 5β-acids has taken place[10]. Bile acids from different species differ chemically in three structural aspects: (1) side-chain structure; (2) stereochemistry of the A/B ring fusion (as mentioned above); and (3) the distribution of the number, position and stereochemistry of hydroxyl groups in the steroid nucleus. Nearly all primary bile acids and bile alcohols, which occur in the less evolved forms of life, have a 7α-hydroxyl group; ursodeoxycholic acid (UDCA) being a notable exception. Most evolved mammalian bile acids have a 5β-configuration with hydroxyl groups at 3α, 7α and 12α, whereas C_{27} bile alcohol sulfates (which increases water solubility) are widespread in nature. These latter are the dominant bile salts of ancient mammalian species, cartilaginous fishes, and some amphibians. The West Indian manatee was the first mammal found to lack bile acids, presumably because it lacks the enzymes required for oxidation of the 26-hydroxy group to a carboxylic acid[11].

Physical characteristics

The presence in bile acid molecules of chemically "non-equivalent" hydroxyl groups (in mammals, commonly at positions 3, 7 and/or 12) and the side chain structure supporting a carboxylic acid group confer them peculiar physical-chemical characteristics, which has made them very attractive building blocks, with repercussions in the design of novel antibiotics[12-14], chiral templates[15], new soft materials[16,17], cation[18] and anion[19,20] receptors, artificial ion channels[21], drug targeting vehicles[22], dendrons[23], molecular baskets[24], scaffolds for combinatorial chemistry[25], new surfactants[26], and others[27,28].

Among the most important physiological properties of bile salts, lipid transport by solubilization and the excretion of cholesterol into the intestinal tract, from which it is poorly absorbed, can be mentioned. These properties are related to their amphipathic nature, which is due to the existence of a hydrophilic side (α-face, concave lower side) and a hydrophobic side (β-face, convex upper side). The hydroxyl groups, oriented towards the α-side (with the exception of the naturally occurring UDCA), and the carboxylic side chain afford them their hydrophilic character. The hydrophobic methyl groups (at C-18 and C-19) are oriented towards the β-side (Figure 1)[29]. As a consequence, they exhibit a great surface activity and in aqueous solutions, they form small aggregates or micelles of usually less than 10 monomers, as long as their concentrations are above a critical value, generally called the critical micellar concentration (CMC). Below the CMC, bile salts behave as 1:1 strong electrolytes, as has been demonstrated from freezing-point measurements[30,31].

The balance between hydrophobic and hydrophilic characters differs markedly among the several molecular species of bile salts. Differences in this balance might account for differences in how bile salts interact with other substances such as, for instance, in the solubilization of phospholipids, cholesterol and other lipids. Over 50 methods have been employed in the



Name	R_1	R_2	R_3	R_4
Cholanic acid	H	H	H	OH
Cholic acid	OH	OH	OH	OH
Chenodeoxycholic acid	OH (α)	OH (α)	H	OH
Deoxycholic acid	OH	OH	OH	OH
Ursodeoxycholic acid	OH (α)	OH (β)	H	OH
Lithocholic acid	OH	H	H	OH
Glicocholate	OH	OH	OH	$NHCH_2COO^-$
Taurocholate	OH	OH	OH	$NHCH_2CH_2SO_3^-$

Figure 1 Structures of the most abundant bile acids in humans, and their glycine and taurine conjugates.

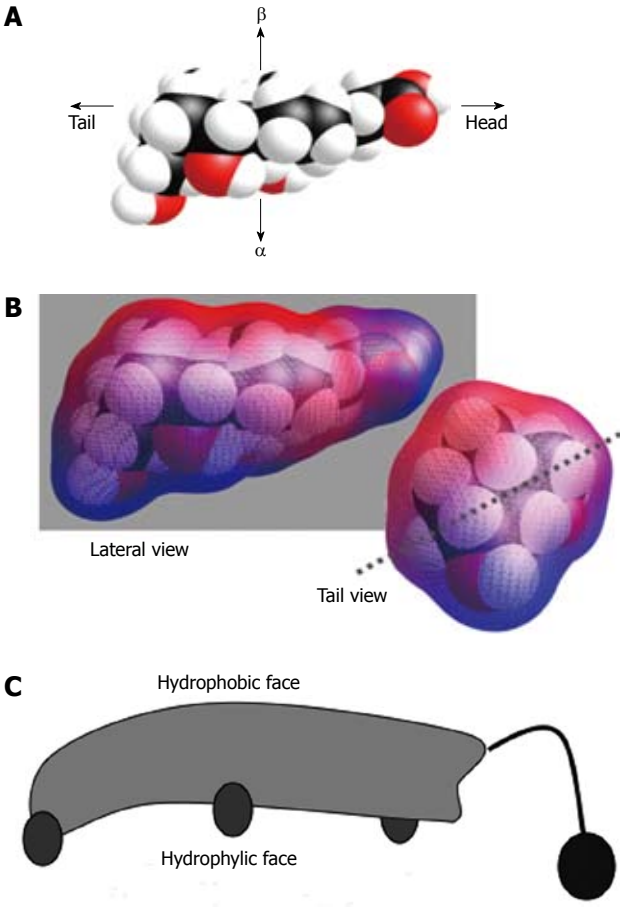


Figure 2 Stereostructure of cholic acid. A: Space-filling model; B: Calculated molecular lipophilic potential[147]. Blue colour shows polar surface and red colour shows apolar surface; C: Cartoon representation (as introduced by Small[148]).

literature to determine the CMC (or pseudo-cmc) values of bile salt solutions, such as the HPLC retention time[32], which accounts in part for the wide range of published values for the CMC[33,34]. The hydrophilicity of the common free and conjugated bile salts decreases in the order UDCA > cholic acid (CA) > chenodeoxycholic acid (CDCA) > deoxycholic acid (DCA) > lithocholic acid (LCA), and taurine-conjugated > glycine-conjugated > free species[35].

These values have been used to predict the cholesterol-solubilizing capacity of all bile salt species, but other physical-chemical and biological properties of individual bile salts also may reflect their hydrophilic-hydrophobic balance[36]. The degree of calcium binding follows the order UDCA < CA < CDCA < DCA < LCA, and taurine-conjugated < glycine-conjugated < free bile salts[35]. In model biles with added gallstones, gallstone masses decrease by addition of bile acids in different degrees, depending on bile acid hydrophobicity (TUDCA > TCA > TCDCA)[37]. However, as noted by Heuman[36], the application of the hydrophilic-hydrophobic balance to determine the physiological properties of bile acids is still an area of controversy. In this respect, Heuman defined a hydrophobic index and extended the method to mixed bile salt solutions[36].

Natalini *et al*[38] have correlated CMC values with hydrophobicity indices, which were determined chromatographically by extrapolating the retention factors back to a virtual pure water-containing mobile phase. Computational methods can also be employed to predict the hydrophobic/hydrophilic balance of bile salts[39]. This balance can be modified by attaching appropriate substituents that enhance either the hydrophilicity or the hydrophobicity of the bile acid, depending on the nature of the organic group. These modifications may be of biological importance. For instance, a series of hydroxycholan-24-amines have been synthesized by modification of the carboxyl group of unconjugated bile acids into a basic moiety[40]. These

Table 1 Minimum and maximum values of CMC in water at 37℃ (in mmol/L) for the sodium salts of major bile acids

Bile acid	Minimum CMC	Maximum CMC
Cholic acid	2.5	29.3
Deoxycholic acid	0.8	70
Chenodeoxycholic acid	3.0	30
Taurocholic acid	1.5	12
Taurodeoxycholic acid	0.6	12
Taurochenodeoxycholic acid	1.25	8

compounds show differential antimicrobial activity against several strains and against fungi[41]. Table 1 summarises the lowest and highest values of CMC reported for the most common bile acids in human bile[33].

PHYSIOLOGY OF BILE ACIDS

Biological functions

Traditionally considered as digestive molecules whose main function is to help in the emulsion and absorption of dietary fats and liposoluble vitamins, bile acids are beginning to be considered more versatile molecules than previously believed. Recent findings have suggested the participation of bile acids in many different functions.

The secretion of bile acids into bile canaliculi generates an osmotic pressure that accounts for the so-called bile-acid-dependent fraction of bile flow[42]. Bile acids stimulate biliary lipid secretion[43] and, due to their physical-chemical properties, are able to form mixed micelles together with biliary phospholipids, which allows the solubilization in bile of cholesterol and other lipohilic compounds. Mixed micelles also account for the emulsion of dietary fat and liposoluble vitamins in the gut, thus helping their absorption. Bile acids also facilitate intestinal calcium absorption[44]. At the intestinal level, bile acids are known to modulate pancreatic enzyme secretion and cholecystokinin release[45]. Moreover, they are potent antimicrobial agents that prevent bacterial over-growth in the small bowel[46].

In the last decade, with the discovery of a specific nuclear receptor able to respond to bile acids, such as the "farnesoid X receptor" (FXR)[47-49], and more recently of their membrane receptor TGR5[50,51], the role of bile acids as signaling molecules with important paracrine and endocrine functions has become evident[52]. Apart from the regulation of their own hepatic synthesis and hepatic and intestinal transport, bile acids are involved in triggering the adaptive response to cholestasis and other insults to the liver[53-55]. Finally, their role in the control of general energy-related metabolism, and more precisely in hepatic glucose handling, has been reported[56].

Synthesis

Bile acids are synthesized from cholesterol (Figure 3). Two main biosynthetic pathways, the so-called "classical" and "alternative" pathways, account for bile acid formation, although several other minor routes have been described, which in some species and situations may also have relevance[57].

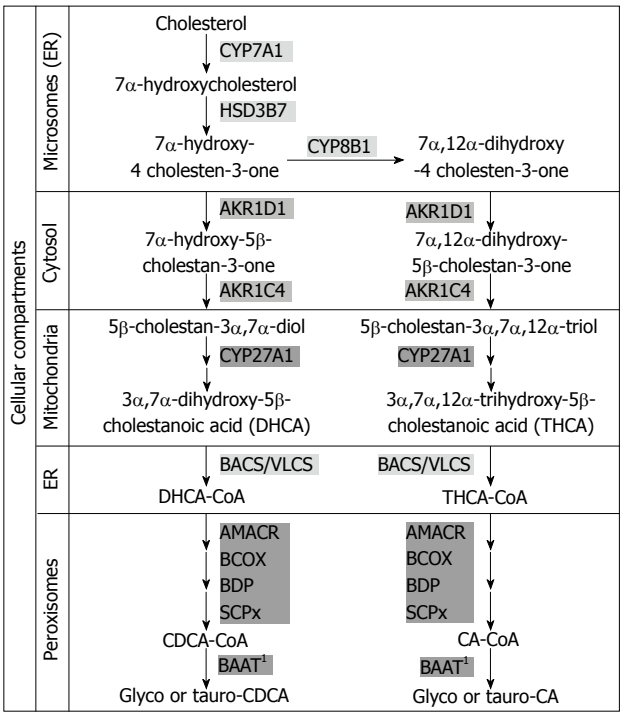


Figure 3 Schematic representation of bile acid synthesis by the classical neutral pathway. AKR1C4: 3α-hydroxysteroid dehydrogenase; AKR1D1: Δ4–3-oxosteroid-5β-reductase; AMACR: Alpha methylacyl-CoA racemase; BAAT: Bile acid; CoA: Amino acid N-acyltransferase ([1]A minor cytosolic fraction does also exist); BACS: Bile acid CoA synthetase; BCOX: Branched-chain acyl CoA oxidase; BDP: D-bifunctional protein hydratase; CYP27A1: Sterol 27-hydroxylase; CYP7A1: Cholesterol 7α-hydroxylase; CYP8B1: Sterol 12α-hydroxylase; HSD3B7: 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase; SCPx: Sterol carrier protein X; VLCS: Very long-chain acyl CoA synthetase; ER: Endoplasmic reticulum.

The classical pathway, also known as the "neutral" pathway because its intermediate metabolites are neutral sterols, is present only in the liver and synthesizes the two primary bile acids in humans: CA and CDCA. This route consists of a cascade of reactions catalyzed by enzymes located at the cytosol, microsomes, mitochondria, and peroxisomes (Figure 3). Extensive descriptions of these reactions and enzymes can be found in several recent reviews[58,59].

In the neutral pathway, the modification of the sterol nucleus of cholesterol precedes the oxidative cleavage of its side chain. It begins with the hydroxylation of cholesterol at C-7, catalyzed by microsomal cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme of the pathway, a cytochrome P450 enzyme localized exclusively in the liver. The resulting 7α-hydroxycholesterol is converted to 7α-hydroxy-4 cholesten-3-one by 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase (HSD3B7), which is also microsomal. The synthesis of CA requires the hydroxylation of 7α-hydroxy-4-cholesten-3-one at the C-12 position, performed by sterol 12α-hydroxylase (CYP8B1), another highly regulated microsomal enzyme[60].

The next steps are catalyzed by two cytosolic enzymes, Δ4–3-oxosteroid-5β-reductase (AKR1D1) and 3α-hydroxysteroid dehydrogenase (AKR1C4), that carry out the reduction of the double bond to obtain 5β-cholestan-3α,7α-diol or 5β-cholestan-3α,7α,12α-triol, the precursors of CDCA and CA, respectively. Mitochondrial sterol 27-hydroxylase (CYP27A1) then oxidizes the side-chain of these precursors by introducing a hydroxyl group to the C-27 position, which is subsequently oxidized to an aldehyde and then to a carboxylic acid. The products, 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), respectively, are activated to their coenzyme A-esters by either bile acid CoA synthetase (BACS) or very long chain acyl CoA synthetase (VLCS), both localized at the endoplasmic reticulum. The resulting cholestanoyl-CoAs are then transported into peroxisomes where the side-chain is shortened by β-oxidation, a process that involves the action of four peroxisomal enzymes (Figure 3).

The final step in bile acid synthesis involves conjugation of the terminal side-chain carboxylic acid with the amino acids glycine or taurine, carried out by the enzyme bile acid CoA: amino acid N-acyltransferase (BAAT). BAAT has been reported to be localized both in peroxisomes and in the cytosol[61], suggesting that peroxisomal BAAT is responsible for conjugation of the newly formed primary bile acids within the peroxisome, while cytosolic BAAT may be involved in the re-conjugation of recycled primary and secondary bile acids previously deconjugated by intestinal bacteria. However, recent studies support the notion that BAAT is mainly a peroxisomal enzyme present in undetectable amounts in the cytosol, and hence deconjugated bile acids returning to the liver need to shuttle to the peroxisome to be re-conjugated[62].

In the alternative biosynthetic pathway for bile acids, side-chain oxidation of cholesterol precedes steroid ring modification. Thus, acidic intermediate metabolites are formed and this pathway is also known as the "acidic" pathway. The first step involves the oxidation of cholesterol to 27-hydroxycholesterol by sterol 27-hydroxylase (CYP27A1), followed by conversion into 7α,27-dihydroxycholesterol by oxysterol 7α-hydroxylase (CYP7B1), a microsomal enzyme specific for this acidic pathway. Since both CYP27A1 and CYP7B1 are expressed in various tissues, and because only the liver has all the required enzymes to accomplish bile acid biosynthesis, these oxidized sterols must be transported to the liver in order to be converted to bile acids. In this pathway, CDCA is the main bile acid formed. The relative contribution of the alternative pathway to overall bile acid synthesis depends on the species considered. In humans, it contributes little to the restitution of daily loss of bile acid (approximately 10%) under normal conditions, but may become the major bile acid biosynthetic pathway in patients with liver diseases[63].

Cholesterol can also be oxidized to 25-hydroxycholesterol and 24-hydroxycholesterol, mainly in extrahepatic tissues such as the brain, an organ with a very high expression of sterol 24-hydroxylase (CYP46A1)[64]. The contribution of these other hydroxylase pathways to overall bile acid synthesis is minor. However, biologically active oxysterols are potent regulators of cholesterol metabolism *via* their nuclear receptor; i.e. the liver X receptor (LXR)[65].

Regulation of bile acid synthesis

Bile acids exert a negative feedback regulation on their own synthesis, in particular by inhibiting CYP7A1 activity[66] and expression[67]. In fact, the cytochrome P450 enzymes CYP7A1, CYP8B1 and CYP27A1 involved in bile acid synthesis are subject to negative feedback regulation by bile acids, which is mainly mediated through the nuclear bile acid receptor FXR. Upon activation by hydrophobic bile acids such as CDCA[68], FXR induces the expression of the small heterodimer partner (SHP) transcriptional repressor. SHP in turn negatively interacts with other transcription factors, liver receptor homolog-1 (LRH-1) and hepatocyte nuclear factor-4α (HNF-4α), that bind to the bile-acid response elements (BAREs) located within the promoter region of the CYP7A1 and CYP8B1 genes[69,70], thus resulting in repression of bile acid synthesis[71,72]. Another FXR-dependent but SHP-independent mechanism for bile acid-induced CYP7A1 down-regulation has been described, involving the secreted fibroblast growth factor 19 (FGF-19) and its receptor FGFR4[73]. Recent studies using liver-specific knock-out mice for FXR and LRH-1 provide strong evidence regarding the importance of the FGF-19/FGFR4 pathway in the control of bile acid synthesis[74,75].

Cholesterol modulates its own catabolism to bile acids, mostly at the transcriptional level. Thus, oxysterols activate LXR, which in turn up-regulates CYP7A1 expression in rat hepatocytes. However, LXR has little or no effect on human CYP7A1[76,77] owing to the lack of an LXR-response element in the promoter of the human *CYP7A1* gene.

Hormones and exogenous compounds may also affect bile acid synthesis. Insulin down-regulates several enzymes of the biosynthetic pathway, such as CYP7A1 and CYP27A1, in different animal species[78], although a dual effect has been described in human hepatocytes[79]. Thyroid hormones induce CYP7A1 gene transcription in rats[80], but the effect of thyroid hormones on the regulation of CYP7A1 in humans is still controversial[81]. Regarding the effects of drugs on bile acid synthesis, both phenobarbital, acting through the nuclear receptor constitutive androstane receptor (CAR)[82], and the antibiotic rifampicin, acting through the pregnane X receptor (PXR)[83], have recently been shown to repress CYP7A1 transcription.

Finally, the activity of CYP7A1 undergoes diurnal variations, paralleled by variations in protein and mRNA levels[84]. Recently, it has been shown that HNF-4α is essential for the maintenance of the diurnal variations in CYP7A1 expression[85]. Also, the circulating levels of FGF-19, which participates in the negative regulation of CYP7A1 expression, show a pronounced diurnal

variation in marked synchronicity with the changes in CYP7A1 activity[86].

Biotransformation

During their intestinal transit, bile acid molecules undergo modifications due to the action of intestinal bacteria. The bile acid metabolism by small intestine microbes consists mainly of de-conjugation and hydroxyl group oxidation. Although ileal bile acid absorption is a very efficient process, some of these molecules (< 1 g/d) escape it and enter the large bowel. The major bile acid modifications in human colon include 7α-dehydroxylation, deconjugation, and oxidation/epimerization of hydroxyl groups at C-3, C-7 and C-12. The deconjugation and oxidation reactions are carried out by a broad spectrum of intestinal anaerobic bacteria. In contrast, bile acid 7α-dehydroxylation is restricted to a limited number of anaerobes representing a small fraction of the total colonic flora[87].

Dehydroxylation at position C-7 is quantitatively the most important bacterial bile acid biotransformation event occurring in the human colon. Bacterial dehydratases of the anaerobic flora from this region attack and remove the hydroxyl group to form 7-deoxy bile acids. Thus, the secondary bile acids DCA (3α,12α-dihydroxy-5β-cholanoic acid) and LCA (3α-hydroxy-5β-cholanoic acid) are formed from CA and CDCA, respectively.

On their side chain, bile acids undergo deconjugation, i.e. enzymatic hydrolysis of the C-24 N-acyl amide bond linking bile acids to their amino acid conjugates. Bile salt hydrolases (BSHs) from the choloylglycine hydrolase family form unconjugated bile acids and free glycine or taurine. Some of these molecules of unconjugated bile acids are taken up by the intestine and return to the liver *via* the portal vein, where they are efficiently taken up and reconjugated during their transit across the hepatocytes toward the bile.

The oxidation and epimerization of the 3-, 7- or 12-hydroxyl groups of bile acids are carried out by the hydroxysteroid dehydrogenases (HSDHs) of intestinal bacteria. Epimerization of bile acid hydroxyl groups is a reversible change in stereochemistry from the α to the β configuration (or *vice versa*), with the generation of a stable oxo-bile acid intermediate. The epimerization of CDCA is the origin of the UDCA (3α,7β-dihydroxy-5β-cholanoic acid) present in the human bile acid pool.

Unlike bile acid oxidation/epimerization, 7α-dehydroxylation appears to be restricted to free bile acids. The removal of glycine/taurine by BSHs is a prerequisite for 7α-dehydroxylation by intestinal bacteria[88]. The deconjugation and 7α-dehydroxylation of bile acids increase their pKa and hydrophobicity, allowing a certain degree of recovery by passive absorption across the colonic epithelium. However, their increased hydrophobicity is also associated with increased toxicity. High concentrations of secondary bile acids in feces, blood, and bile have been linked to the pathogenesis of cholesterol gallstone disease and colon cancer[89].

Enterohepatic circulation

The interactions of bile acids with the intestine, including ileal bile acid transport and its regulation, have been reviewed in a separate paper of this series[90]. Here we shall briefly comment on the major points of this aspect of bile acid physiology. Bile acid molecules are mostly confined to the territories of the so-called enterohepatic circulation, which includes the liver, the biliary tree, the intestine and the portal blood with which bile acids are returned to the liver. Upon completion of their digestive tasks, most intestinal bile acids (95%) are recovered by active transport in the intestine, mainly in the ileum. Active uptake of bile acids at the apical membrane of intestinal epithelial cells is performed by the apical sodium-dependent bile acid transporter (ASBT, gene symbol *SLC10A2*). This carrier is a symporter able to co-transport two sodium ions together with one molecule of bile acid[91]. For a long time, the efflux of bile acids from intestinal cells across the basal membrane has been a matter of controversy. The currently accepted concept is that this process is mainly accounted for by the heterodimeric organic solute transporter alpha and beta (OSTα-OSTβ)[92].

Albumin-bound bile acids that reach the liver mainly *via* the portal blood but also, although to a lesser extent, *via* the hepatic artery, are efficiently removed by transport proteins located at the sinusoidal membrane of hepatocytes. The first-pass extraction fraction ranges from 50% to 90%, depending on the bile acid structure[93]. The uptake of conjugated bile acids is largely sodium-dependent and is performed by the Na-taurocholate co-transport polypeptide (NTCP, *SLC10A1* gene)[94]. Sinusoidal sodium-independent bile acid uptake also occurs. This process is carried out by members of the family of organic anion transporting polypeptides (OATP), mainly the OATP1B1 and OATP1B3 isoforms[95]. In the overall process of bile acid transport from blood to bile, canalicular secretion is the limiting step. This transport for monoanionic amidated bile acids, which constitute the majority of secreted bile acids, is ATP-dependent and is mainly performed by the bile salt export pump (BSEP, gene symbol *ABCB11*)[96]. Highly hydrophobic bile acids, such as LCA, can be sulfated in human hepatocytes as a means of reducing its toxicity by increasing its water-solubility. Bile acids conjugated with sulfate or glucuronic acid are dianionic and are transported by other canalicular pumps, such as MRP2 (*ABCC2* gene)[97] and BCRP (*ABCG2* gene)[98].

The high specificity of these hepatic and intestinal carrier proteins for bile acids accounts for the low levels of these compounds in peripheral blood, commonly below 10 μmol/L in healthy subjects[99].

PATHOPHYSIOLOGY OF BILE ACIDS

Defects in bile acid synthesis

Defects in bile acid synthesis are uncommon genetic disorders that account for approximately 1%-2% of cholestatic disorders in children[100]. The inheritance of

Table 2 Inborn defects in bile acid synthesis and biotransformation

Impaired process	Defect localization	Consequences
Sterol ring modification	Cholesterol 7α-hydroxylase (CYP7A1)	Increased hepatic cholesterol. In adults, LDL hypercholesterolemia and cholesterol gallstones
	Oxysterol 7α-hydroxylase (CYP7B1)	Accumulation of monohydroxyl bile acid species with marked cholestatic and hepatotoxic capabilities. Severe neonatal liver disease
	3β-Hydroxy-C27-steroid dehydrogenase/somerase (HSD3B7)	Cholestatic jaundice and malabsorption of lipids and lipid-soluble vitamins
	δ-4-3-Oxosteroid 5β-reductase (AKR1D1)	Accumulation of δ-4-3-oxo- and allo(5α-H)-bile acids. Liver disease rapidly progressing to liver failure
Side-chain modification	27-Hydroxylase (CYP27A1)	Cerebrotendinous xanthomatosis
	25-Hydroxylase (CH25H)	Low levels of primary bile acids in serum and increased urinary excretion of typical bile alcohols
	α-Methylacyl-CoA racemase (AMACR)	High concentrations of (25R) trihydroxy-cholestanoic acid in urine, bile, and serum
	Complete or partial absence of peroxisomes	Zellweger syndrome Infantile Refsum disease Neonatal adrenoleukodystrophy Hyperpipecolic acidemia
	Altered peroxisomal enzymes	Pseudo-Zellweger syndrome Pseudo-neonatal adrenoleukodystrophy X-linked adrenoleukodystrophy
Bile acid amidation	Bile acid acyltransferase (BAAT)	Absence of taurine or glycine conjugates. Enhanced proportion of sulfate and glucuronide conjugates
	Bile acid-CoA ligase?	Absence of taurine or glycine conjugates. Enhanced proportion of sulfate and glucuronide conjugates

these defects is autosomal and recessive. The resulting liver diseases vary from mild to severe, depending on the particular alteration. The most common clinical presentation is progressive cholestasis of infancy, although other clinical manifestations, such as advanced liver disease at birth, neonatal hepatitis or the development of liver disease in later childhood, can also occur. When the enzymatic defect results in an accumulation of toxic monohydroxylated and/or unsaturated oxo-bile acids, many of which are cholestatic[101], the progression of liver disease is usually rapid. Recent evidence suggests that certain cholestatic liver diseases in adults may also be due to an inherited defect in bile acid biosynthesis[102].

Diagnosis is accomplished by analysis of the profile of bile acid species and their precursors and/or metabolites in body fluids, using laboratory techniques such as fast atom bombardment-mass spectroscopy and gas chromatography-mass spectroscopy. Early diagnosis is critical for these patients, because several of these disorders can be successfully treated with the dietary addition of bile acids. This has a dual purpose: first, to replace the essential primary bile acids absent, and second, to down-regulate bile acid synthesis by negative feedback inhibition, thus reducing the production of abnormal toxic intermediate metabolites by hepatocytes bearing the defect.

As will be commented below in detail, inborn errors affecting the enzymes involved both in the modification of the sterol nucleus and the side-chain, as well as in side-chain amidation, have been identified (Table 2). Moreover, the absence or impaired function of peroxisomes also results in alterations in bile acid metabolism that accompany the other signs characterizing each syndrome (Table 2).

Defects in the modification of the sterol nucleus

At least four inborn errors affecting enzymes that modify the sterol rings have been identified. Three of them are associated with progressive liver disease.

Defect in cholesterol 7α-hydroxylase: The defect in the key enzyme of the classical pathway of bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1), has been associated with a decrease in bile acid production *via* the classical pathway, which is compensated by activation of the alternative acidic pathway[103]. In these individuals, hepatic cholesterol contents are increased and, in adults, LDL hypercholesterolemia and cholesterol gallstones are commonly present. However, usually there is no evidence of liver disease.

Defect in oxysterol 7α-hydroxylase: A defect in the conversion of 27-hydroxy-cholesterol to 7α,27-dihydroxy-cholesterol due to a deficiency in oxysterol 7α-hydroxylase (CYP7B1), an enzyme specifically involved in the acidic pathway, causes severe neonatal liver disease. This is probably due in part to the accumulation of monohydroxyl bile acid species, with marked cholestatic and hepatotoxic capabilities[104]. This defect, resulting from a mutation in the gene, reveals the importance in humans of this alternative pathway in early life.

Defect in 3β-hydroxy-C27-steroid dehydrogenase/isomerase: This enzyme catalyzes the oxido-reduction of the 3β-hydroxyl group of 7α-hydroxycholesterol. Its deficiency is the most common defect in bile acid synthesis[105,106]. Individuals with autosomal recessive mutations in the encoding gene, *HSD3B7*, fail to

synthesize bile acids normally and develop a form of progressive liver disease characterized by cholestatic jaundice and malabsorption of lipids and lipid-soluble vitamins.

Defect in δ-4-3-oxosteroid 5β-reductase: The absence of this cytosolic enzyme results in a lack of the ability to reduce the double bond between C-4 and C-5 of the sterol A-ring, and thus to convert 3-oxo intermediates into the corresponding 3α-hydroxyl products, an essential step in major bile acid synthesis. This defect results in a markedly reduced primary bile acid synthesis and a concomitant accumulation of δ-4-3-oxo- and allo(5α-H)-bile acids[107]. A clinical presentation resembling that of neonatal hepatitis is typical, together with rapidly progressive liver disease and liver failure in infancy. Treatment with bile acid replacement therapy provides beneficial results.

Defects in the modification of the side-chain

Several inborn errors affecting single enzymes involved in the modification of the cholesterol side-chain to produce C_{24} bile acids have been identified. Additionally, because β-oxidation of the side-chain occurs in peroxisomes, peroxisomal disorders can also affect bile acid synthesis, accompanying other manifestations typical of each syndrome[108].

Defect in sterol 27-hydroxylase: A mitochondrial sterol 27-hydroxylase (CYP27A1) deficiency accounts for the development of so-called cerebrotendinous xanthomatosis (CTX)[109]. Regarding the biosynthesis of bile acids, this defect specifically interferes with the initial modifications of the cholesterol side-chain, resulting in downstream production of bile alcohols and a decreased synthesis of primary bile acids[110,111]. In general, CTX must be considered a progressive lipid storage disease characterized by diarrhea (the earliest clinical manifestation, affecting approximately 75% of affected infants), cataract (appearing in the first decade of life), tendon xanthomas (adolescent- to young adult-onset), and neurologic alterations, such as dementia, psychiatric disturbances, pyramidal and/or cerebellar signs, and seizures (adult-onset). Owing to the formation of deposits of cholesterol and cholestanol, xanthomas appear on the Achilles tendon, the extensor tendons of the elbow and hand, the patellar tendon, and the neck tendons, but also in the lung, bones, and central nervous system.

Defect in 25-hydroxylase: An inborn error in sterol 25-hydroxylase (CH25H), which is involved in the alternative pathway for bile acid side-chain synthesis, has been suggested to account for the bile acid profile that is found in some cases of neonatal hepatitis syndrome. This is characterized by the presence of low levels of normal primary bile acids in serum and increased urinary excretion of typical bile alcohols[112].

Defect in alpha methylacyl-CoA racemase: Alpha methylacyl-CoA racemase (AMACR) deficiency is a recently described defect in bile acid side-chain oxidation[113,114]. This peroxisomal enzyme catalyzes the conversion of (25R) trihydroxy-cholestanoic acid (THCA) to its 25S isomer, a step that is essential for the subsequent peroxisomal β-oxidation to primary bile acids to be initiated. High concentrations of (25R) THCA are found in the urine, bile and serum of these patients.

Peroxisomal defects: Disorders in peroxisomal biogenesis (absence or diminished numbers of peroxisomes) and specific enzymatic defects in peroxisome-based lipid oxidation include a group of diseases (Table 2) that present an important phenotypical overlap, with variability in the type of liver disease developed[115]. Altered serum bile acids in patients with peroxisomal disorders have been described[116]. The cerebro-hepato-renal syndrome of Zellweger is probably the condition in which hepatic function is most affected; atypical mono-, di- and tri- hydroxy C-27 bile acids with low amounts of primary bile acids are present in this disease[117,118].

Apart from AMACR, other peroxisomal enzymes involved in the beta-oxidation of the bile acid side-chain are branched-chain acyl-CoA oxidase, D-bifunctional protein and sterol carrier protein X (SCPx). Deficiencies in these enzymes, associated with abnormalities in bile acid synthesis, have also been reported[108].

Defects in bile acid amidation

Defective bile acid conjugation, which is characterized by a complete absence of glycine and taurine conjugates of bile acids in biological fluids and a predominance of unconjugated CA, with small proportions of sulfate and glucuronide conjugates, has been reported[119]. Fat-soluble vitamin deficiency is severe. The authors proposed a defect in bile acid-CoA ligase, because no CA-CoA derivatives were detected in any biological fluids, although no genetic analyses were performed in that study. Until now, alterations in *SLC27A5* gene encoding for VLCS or bile acid-CoA ligase have not been described in humans, therefore deficiency of this enzyme remains a hypothetical disorder. However, as mice with deleted *SLC27A5* do have the expected phenotype[120], the possibility of the existence of the corresponding metabolic disorder in humans can be expected.

More recently, a similar biochemical phenotype caused by a homozygous mutation in BAAT has been reported in Amish individuals with familial hypercholanemia, pruritus, and fat malabsorption[121].

Defects in bile acid transport

Progressive familial intrahepatic cholestasis (PFIC) type 1 (Byler disease), type 2 and type 3 are genetic disorders of bile secretion in which the fundamental abnormality is the direct or indirect defective hepatobilary transport of bile acids and/or phospholipids. Inborn errors of biliary canalicular transport systems will be the subject of a separate paper of this series and have been previously

reviewed by others[122,123].

Among these diseases, PFIC type 2 is due to primarily impaired bile acid transport. In these patients, high levels of serum bile acids, together with severe progressive liver disease, are found. PFIC type 2 is caused by a mutation in the bile salt export pump (BSEP, gene symbol *ABCB11*)[124,125], the main agent responsible for the ATP-dependent secretion of monoanionic bile acids across the canalicular membrane[96].

The less severe variant of PFIC type 2 is benign recurrent intrahepatic cholestasis (BRIC) type 2. This is a mild condition characterized by intermittent crises of cholestasis without permanent liver damage. BRIC type 2 is also caused by mutations in *ABCB11*[126].

Mutations in the *BSEP* gene have also been related to the aetiology of intrahepatic cholestasis of pregnancy[127,128].

BILE ACIDS IN PATHOLOGY

Bile acids as deleterious agents

Owing to their amphipathic characteristics, bile acids may behave as detergent molecules, which in many cases is the primary cause of bile acid-induced damage when they accumulate in the liver and other organs[129]. In the cholestatic condition known as PFIC type 3, a defect in MDR3 (gene symbol *ABCB4*) occurs. MDR3 is the flopase involved in the translocation of phospholipids, mainly phosphatidylcholine, from the inner to the outer leaflet of the canalicular membrane[130]. The presence in the biliary lumen of bile acids, whose detergent ability is not buffered by phosphatidylcholine, causes attack and disruption by solubilizing the lipidic components of the apical membranes in hepatocytes and biliary epithelial cells. As a side effect, this results in an increased release of gamma-glutamyltranspeptidase, whose serum levels are higher than normal.

Elevated intracellular concentrations of bile acids, such as those attained in cholestasis, have been related to oxidative stress[131] and apoptosis, both in adult and fetal liver[132]. Bile acids may induce apoptosis both by directly activating the Fas death receptor[133] and by inducing oxidative damage that causes mitochondrial dysfunction, which in turn may trigger apoptosis[134,135].

Finally, a relationship between bile acids and cell proliferation also exists. Some bile acid species have been shown to modulate DNA synthesis during liver regeneration after partial hepatectomy in rodents[136,137], and the regenerative process is dependent on bile acid signaling through the nuclear receptor FXR[138]. Teratogenic[139] and carcinogenic[140] effects of the more hydrophobic bile acids have been reported. Thus, a role of bile acids in the etiology of cancer at different sites - colon, esophagus, or even non-digestive tissues such as breast - has been suggested[141,142]. Moreover, it has recently been shown that mice lacking FXR spontaneously develop liver tumours[143,144].

Secondary alterations in bile acid homeostasis

The normal hepatic synthesis and enterohepatic circulation of bile acids are altered in some pathological conditions. This can indeed be expected in chronic liver diseases such as hepatitis or cirrhosis, which indirectly impair bile secretion, but this is also the case in other pathologies that do not directly affect hepatocyte secretory function, but in which changes in bile acid metabolism secondary to the primary disease have been described. This group of diseases includes cystic fibrosis[145] and diabetes mellitus[146].

CONCLUSION

From the results obtained over the past three decades, it is becoming evident that bile acids can no longer be considered as simple detergent compounds that are useful in digestive processes. The list of their physiological roles, as well as that of the pathological processes in which they are involved either as etiological agents, mediators of the pathogenic process, or simply affected by disease-induced changes in the liver or the intestinal handling of these steroids, is long and still not complete. Moreover, owing to their peculiar physical-chemical and biological characteristics, the huge potential usefulness of bile acids in the development of pharmaceutical approaches as well as their use as natural drugs or as the basis for the synthesis of novel semisynthetic drugs is encouraging many different groups worldwide to invest efforts in this direction. There is no doubt that many new concepts, pharmaceutical tools and pharmacological uses of bile acids and their derivatives will emerge in the near future.

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TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Excretion of biliary compounds during intrauterine life

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Abstract

In adults, the hepatobiliary system, together with the kidney, constitute the main routes for the elimination of several endogenous and xenobiotic compounds into bile and urine, respectively. However, during intrauterine life the biliary route of excretion for cholephilic compounds, such as bile acids and biliary pigments, is very poor. Although very early in pregnancy the fetal liver produces bile acids, bilirubin and biliverdin, these compounds cannot be efficiently eliminated by the fetal hepatobiliary system, owing to the immaturity of the excretory machinery in the fetal liver. Therefore, the potentially harmful accumulation of cholephilic compounds in the fetus is prevented by their elimination across the placenta. Owing to the presence of detoxifying enzymes and specific transport systems at different locations of the placental barrier, such as the endothelial cells of chorionic vessels and trophoblast cells, this organ plays an important role in the hepatobiliary-like function during intrauterine life. The relevance of this excretory function in normal fetal physiology is evident in situations where high concentrations of biliary compounds are accumulated in the mother. This may result in oxidative stress and apoptosis, mainly in the placenta and fetal liver, which might affect normal fetal development and challenge the fate of the pregnancy. The present article reviews current knowledge of the mechanisms underlying the hepatobiliary function of the fetal-placental unit and the repercussions of several pathological conditions on this tandem.

Key words: Bile acids; Bilirubin; Foetus; Liver; Placenta

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INTRODUCTION

In the adult liver, most cholephilic organic anions are taken up from the portal blood by hepatocytes across the basolateral plasma membrane by sodium-dependent and -independent carriers (Figure 1). These are members of two groups of proteins: (1) the organic anion-transporting polypeptides family (OATP, gene symbol *SLCO*), whose isoforms OATP1B1, OATP1B3 and to a lesser extent OATP1A2[1], play a major role in the uptake of cholephilic compounds by human hepatocytes; (2) the Na^+-taurocholate-cotransporting polypeptide (NTCP, *SLC10A1*)[2]. Several members of the organic anion transporter (OAT) and organic cation transporter (OCT) family (gene symbol *SLC22A*) collaborate in the uptake of a large variety of organic molecules by the liver.

The secretion of cholephilic compounds into bile is accounted for by export pumps located at the canalicular plasma membrane. These are proteins belonging to the ATP-binding cassette (ABC) superfamily, which, in this region of the hepatocyte include the P-glycoprotein or multidrug resistance protein (MDR1; *ABCB1*), able to transport organic and inorganic cations[3]; the sister of P-glycoprotein or bile salt export pump (BSEP; *ABCB11*), which constitutes the main secretory system for bile acids[4]; the isoform 2 of the multidrug resistance-associated protein (MRP2; *ABCC2*), which exports conjugated forms of bilirubin, bile acids and xenobiotics[5,6], and the breast cancer resistance protein (BCRP; *ABCG2*), able to export sulfated steroids, which probably include bile acids[7].

In normally functioning healthy adult livers, at least as far as the excretion of cholephilic compounds into bile is concerned, the expression levels of MRP1 (*ABCC1*) and

MRP3 (*ABCC3*) at the basolateral membrane of hepatocytes is low[8,9]. However when the biliary excretory route is impaired, such as in cholestasis or endotoxemia, cholephilic compounds accumulate in hepatocytes, inducing an up-regulation of basolateral export pumps[10-12]. This acts as an adaptative response to reduce the cytotoxic effects of cholephilic compounds by pumping them back to the systemic circulation and accounts for an increased elimination of these substances into urine[13].

During pregnancy, owing to the immaturity of the fetal hepatobiliary excretory function, the existence of an alternative mechanism for the detoxification of cholephilic compounds produced by the fetus is required. The placenta, in collaboration with the maternal liver, carries out this function, which is very important for maintaining low bile acid and bilirubin levels in the fetal compartment. Moreover, the placenta also protects the fetal compartment, at least to a certain extent, from potentially toxic compounds coming from the maternal blood[14]. When the fetal-maternal homeostasis is altered, as happens during intrahepatic cholestasis of pregnancy, and these molecules accumulate in the *conceptus*, the consequences can be as serious as stillbirth and fetal death[15].

THE HEPATOBILIARY EXCRETORY FUNCTION DURING INTRAUTERINE LIFE

Fetal bile acid synthesis and maturation of the enzyme equipment required for bile acid and bile pigment metabolism precede the development of an efficient biliary-secretory system. Thus, although during intrauterine life bile acids are not required for digestive purposes, the fetal liver is able, from very early on during gestation, to synthesize primary bile acids, mainly cholic acid and chenodeoxycholic acid from cholesterol. Indeed, these two molecules are the major components of the human fetal bile acid pool[16-17]. The fetal bile acid pool is also characterized by the presence of molecular species with hydroxyl groups in positions that are unusual in bile acids found in adults. These are C-1, C-4 and C-6[18], which convert the molecule into a more hydrophilic one. This is believed to protect the fetal liver against the cytotoxic effect of less polar bile acid species when detoxification pathways are poorly developed. Another important characteristic of the fetal bile acid pool is the existence of bile acids with "flat" structures, accounted for by the presence of Δ4 or Δ5 insaturations or the alpha configuration of a hydroxyl in C-5[18]. Although the fetal gut is germ-free, the bile acid pool contains small amounts of secondary bile acids, such as deoxycholic acid and lithocholic acid, together with tertiary bile acids, such as ursodeoxycholic acid. This is probably due to placental transfer of these compounds from the maternal circulation[19].

Data collected from both rats[20] and humans[16,19,21] have revealed that serum bile acid concentrations are higher in fetuses than in their mothers, and that the composition of the bile acid species in both compartments is different. This has been explained in terms of the selective transplacental transfer of these cholephilic compounds[19], together with a different degree of maturation of the enzymatic machinery involved in bile acid metabolism[22]. The recently described role of bile acids as signaling molecules with several endocrine and paracrine functions[23] might account for the yet unknown physiological meaning of the early synthesis and special composition of the bile acid pool in fetuses.

From early gestation, the fetus also produces biliary pigments. The green pigment biliverdin, mainly the IXα isomer, is generated by cleavage of protoporphyrin IX by heme oxygenase[24,25] and is reduced to the golden pigment bilirubin IXα by the enzyme biliverdin reductase. The high production of bilirubin by the fetal liver, together with the still low activity in this organ of glucuronosyl transferase, the enzyme that produces more polar glucuronide conjugates to facilitate biliary excretion in adults, account for the higher concentrations of the unconjugated pigment in fetal serum than in maternal serum[19,26].

For many years, these bile pigments were considered mere waste products from heme metabolism, and the biological advantage of the conversion of the water-soluble and non-toxic compound biliverdin into the poorly water-soluble and neurotoxic compound bilirubin was not understood. Since the efficacy of biliverdin and bilirubin glucuronide transfer across the placenta is very poor, it was suggested that the formation of bilirubin from biliverdin may play a role in facilitating the elimination of heme-derived pigments in utero[27]. During the last decade, however, different studies have demonstrated the ability of bilirubin to protect cells against free radical damage both *in vitro* and *in vivo* in several tissues[24,28,29]. A recent work carried out by our group[30] revealed that, up to a certain degree of accumulation of bilirubin (below toxic levels), this pigment may help to protect the placental-fetal unit from maternal cholestasis-induced oxidative stress. Together with its direct antioxidant properties, bilirubin is also able to induce the expression of antioxidant systems. Thus, the current concept is that, when maintained in the physiological non-toxic range, bilirubin must be considered a beneficial compound[31].

In spite of the immaturity of fetal bile secretion, small amounts of bile acids have been detected in the gallbladder bile collected from human fetuses obtained from abortions older than 12 wk of age[32]. Regarding bile pigments, although the IXβ isomer of bilirubin constitutes only a small fraction of the total amount produced in the fetus[33], this more water-soluble isomer is the most abundant isomer found in fetal gallbladder bile and meconium[34,35]. The reason for this is two-fold: (1) bilirubin IXβ cannot easily cross the placenta; and (2) it can be excreted into bile without previous conjugation with glucuronic acid[36].

The fact that the expression of export pumps, such as Mrp2 and Bsep, only appears in rat fetal liver in the last third of gestation[37,38] is probably the cause of the low efficiency of this route of excretion during pregnancy. As previously commented, the serum levels of bile acids and bile pigments are higher in the fetus than in the mother. It is not known how cholephilic organic anions generated by the fetal liver reach the sinusoidal blood, but because some OATPs may act as bi-direction-

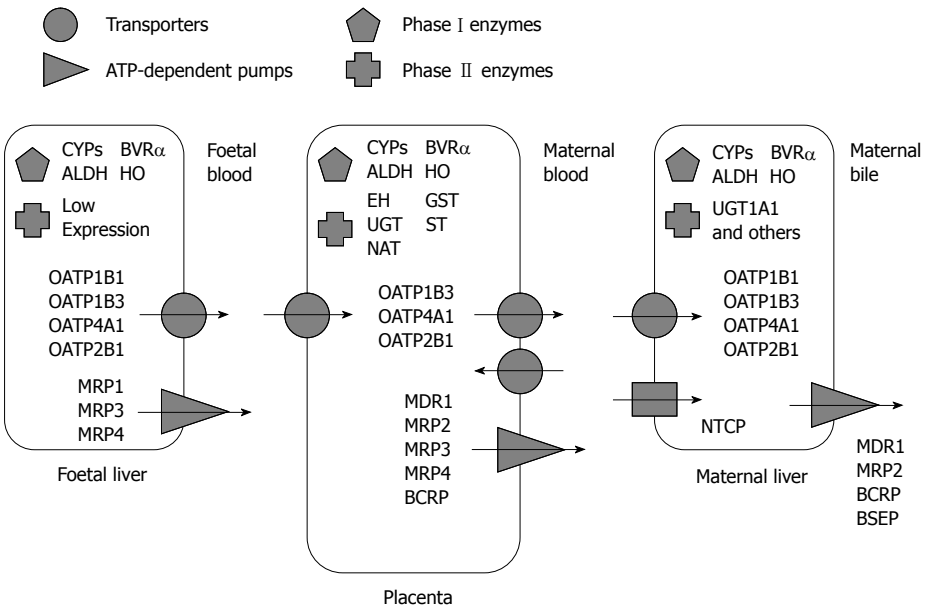

Figure 1 The foetal liver-placenta-maternal liver excretory pathway. Schematic representation of transporters (NTCP, sodium-taurocholate co-transporting polypeptide; OATP: Organic anion-transporting polypeptide), ATP-dependent pumps (BCRP: Breast cancer resistance protein; BSEP: Bile salt export pump; MDR: Multidrug resistance protein; MRP: Multidrug resistance-associated protein), and phase I (ALDH: Aldehyde dehydrogenase; BVR: Biliverdin reductase; CYP: Cytochrome P450 enzyme; HO: Heme oxygenase) and II (EH: Epoxide hydrolase; GST: Glutathione-S-transferase; NAT: N-acetyltransferase; ST: Sulfotransferase; UGT: UDP-glucuronosyl transferase) enzymes involved in excretion of biliary compounds during intrauterin life.

al transporters[39-41], they are good candidates for carrying out this function. Moreover, the expression of several OATP isoforms has been detected in rat fetal liver[38,42]. However, the abundance of these transporters in fetal liver is much lower than in adult liver, except for Oatp4a1[43] and its human orthologue OATP4A1[44]. Another possibility is that cholephilic compounds may exit the fetal liver *via* ATP-dependent pumps of the ABC family located in the basolateral plasma membrane. Supporting this concept is the higher expression of Mrp1 and Mrp3 in fetal than in maternal rat liver[38,42].

The accumulation of bile acids in fetal serum can have serious consequences, depending on the magnitude of the hypercholanemia[45]; in the most severe cases there is an increased risk of stillbirth and perinatal mortality[46], while in less severe conditions, maternal hypercholanemia can affect normal fetal development, the liver being one of the tissues most affected[47]. In fact, in a laboratory animal model of maternal hypercholanemia, the repercussions on fetal hepatobiliary function, although reversible, are maintained in young animals[20], and are characterized by a partial impairment in the ability of the liver to secrete organic anions, whereas the bile acid-induced biliary secretion of phospholipids, but not cholesterol, is increased[20,48].

The fetal kidney is able to secrete small amounts of organic anions into the amniotic fluid[17]. This, together with the detection of ABC proteins in the apical membrane of the yolk sac, has led to the suggestion that fetal membranes provide an additional route to protect the fetus against endogenous and xenobiotic compounds[49]. However, owing to the immaturity of the fetal renal system, the importance of this route in excreting cholephilic compounds during gestation is low[50-52].

ROLE OF THE PLACENTA IN THE EXCRETION OF BILIARY COMPOUNDS

Based on the foregoing it is clear that in contrast to what happens in the adult where the hepatobiliary system with the collaboration of the kidney are responsible for the biotransformation and elimination of bile acids, biliary pigments, drugs and food components, the main route for the elimination of these compounds during intrauterine life is their transfer to the mother across the placenta. Later on, the biotransformation and elimination into feces and urine is carried out by the maternal liver and, to a lesser extent, by the maternal kidney, respectively.

Excretion of bile acids

As mentioned above, there is a transplacental gradient for bile acids in the fetal-to-mother direction, except for secondary and tertiary bile acids, which are more abundant in maternal serum[19]. Several experimental lines of evidence suggest that simple diffusion is not the main mechanism by which these organic anions cross the human placenta[53]. In fact, ATP-dependent mechanisms account for the vectorial transfer of these compounds in the fetus-to-mother direction[54]. This has important implications, because in situations of maternal hypercholanemia there is only a moderate increase in bile acid concentrations in fetal serum[47].

The human placenta is of the haemochorial type, i.e. only the endothelium of chorionic vessels, the stroma of chorionic villi and the trophoblast layer separate the fetal and maternal blood. This means that in order to eliminate fetal metabolic by-products across the placenta, they must cross these three components of the placental barrier. Once in the maternal blood, most foetal bile acids are eliminated in bile by the maternal liver and excreted into feces. Regarding this task, the maternal kidney only contributes slightly to the excretion of sulphated and glucuronidated species[55].

For several years there has been functional evidence for a mediated transport of cholephilic organic anions at both poles of human and rat trophoblasts[56]. Functional studies carried out on isolated human trophoblast mem-

brane vesicles have suggested the presence of an anion exchanger transport system for the uptake of bile acids across the basal membrane of trophoblasts[53]. The trans-activation of this transport system with bicarbonate[57], the fact that substrate specificity is not restricted to bile acids[58] and the different affinities found for bile acid species depending on the number of hydroxyl groups and amidation[59] have led to the speculation that in the fetal-side membrane of the trophoblast there are proteins, probably belonging to the OATP family, that could be responsible for the uptake of organic anions by the trophoblast from fetal blood.

With respect to the opposite pole of the polarized epithelial trophoblastic cells, functional studies using plasma membrane vesicles have demonstrated that the transfer of bile acids toward the maternal circulation is dependent on ATP hydrolysis, in both human[54] and rat[60] placentas. However, it has been suggested that in the absence of ATP, bile acids could also cross this membrane by electrogenic-facilitated diffusion[61] and/or anion exchange[62]. The ATP-dependent system has higher substrate affinity, while the ATP-independent system has greater capacity[54]. These data suggest that in the apical membrane of trophoblasts, ABC proteins may be involved in pumping out bile acids towards the mother and, that proteins of the OATP family may participate in the ATP-independent component of this transport.

Concerning human OATPs, the mRNA of OATP1A2, OATP1B1 and OATP1B3 was detected in human placenta using real-time quantitative PCR[63,64]. The expression levels of OATP1A2 and OATP1B1 were shown to be very low at term, although they were detected at higher levels early during gestation[65]. Both OATP2B1 and OATP4A1 were also highly expressed in human placenta[44,66]. However, the former is not believed to be involved in the transport of bile acids[67], while the latter, which is considered to be a thyroid hormone carrier, is able to transport several bile acids[44].

Regarding the expression of Oatps in rat placenta, several isoforms have been detected. Under normal physiological circumstances, mRNA expression levels at term were low for Oatp1a1, Oatp1b4 and Oatp1b2, but high for Oatp4a1[42]. However, maternal cholestasis induces an up-regulation of these transporters, which is further enhanced when pregnant rats are treated with UDCA[68]. In addition, Oatp2b1 is also present in rat placenta and its ability to transport taurocholate has been described[69].

Little is known about the sub-tissue and sub-cellular localization of OATPs in the placenta. It has been suggested that OATP2B1 would be localized in the basal plasma membrane of the trophoblast[66], whereas OATP4A1 has been detected in the apical plasma membrane[44].

Regarding ABC proteins, several members of this superfamily are expressed in the placenta. The main system accounting for bile acid excretion into bile, BSEP, has been detected at very low levels in human and rat placentas at term[42,64,70], but at higher levels during the first trimester of pregnancy in humans[65].

Some members of the MRP family with the ability to transport biliary compounds - MRP1, MPR2, MRP3 and MRP4 - have been identified in human placenta[64,71]. The mRNA expression levels of MRP1 are higher in placenta than in liver; the abundance of MRP2 in the placenta is low compared to liver, and the expression levels of MRP3 and MRP4 are similar and low in both tissues, respectively[64].

The available data for the rat orthologues Mrp1, Mrp2, Mrp3 and Mrp4 suggest that they have similar expression patterns as those described for human isoforms[68]. Moreover, it has been observed that, at least in rats, there is a strong up-regulation of these transporters during maternal cholestasis, which can contribute to the protection of the fetus against the high concentrations of bile acids and biliary pigments existing on the maternal side of the placenta under these circumstances[68].

The cellular localization of some of these proteins in the placenta is controversial. MRP1, MRP2 and MRP3 have been detected by immunofluorescence and Western blotting in the apical membrane of the syncytiotrophoblast[71], and MRP1 is also expressed in fetal blood vessels[71], and in the basal membrane of the syncytiotrophoblast[72].

Another important member of the ABC family is the breast cancer resistance protein (BCRP, *ABCG2*), also known as ABC placental protein (ABCP) due to its high expression in this organ[73]. This protein is able to export a broad range of substrates, which have been reported to include bile acids[7]. BCRP has been detected in the apical membrane of trophoblasts and in fetal vessels[64,74]. Both the mRNA and protein levels of Bcrp are higher in rodent placenta during mid-gestation but decrease at term[75].

Excretion of biliary pigments

The endothelium of chorionic vessels and the syncytiotrophoblast are exposed to high concentrations of hemoglobin through their direct contact with fetal and maternal blood, respectively. Hemoglobin and free heme can undergo auto-oxidation to produce superoxide (O_2^-) and H_2O_2, which in turn promote the formation of other highly reactive and damaging radical species. These include lipid peroxides and the very reactive hydroxyl radical if trace amounts of free iron are available[76]. Heme can be degraded either enzymatically or chemically. Both mechanisms utilize molecular oxygen (O_2) and require a reducing agent. In the reaction catalyzed by heme oxygenase (HO), NADPH is the source of the reducing equivalent[77].

HO is a microsomal enzyme that induces the cleavage of heme, a pro-oxidant, to produce the biliary pigment biliverdin, iron and carbon monoxide (CO)[77]. There are three HO isoenzymes: HO-1 is a 32 kDa protein also known as heat-shock protein (HSP) 32, which is expressed at high levels in spleen and liver. HO-1 can be induced by several stimuli including hypoxia and hyperoxia[78,79]. The induction of HO-1 was coupled to the synthesis of the iron-sequestering protein, ferritin[80]. Ferritin avidly binds iron and interrupts the redox cycling of iron, thereby preventing iron from being useful as a catalyst for oxidant stress[81]. Subsequent studies

have demonstrated that the induction of HO-1 is also coupled to the synthesis of iron-exporting proteins and hence a critical role of HO-1 in maintaining iron homeostasis *in vivo* has been suggested[82].

HO-2 is a 36 kDa protein that is widely distributed in tissues throughout the body, where it is constitutively expressed and not readily inducible[83]. HO-2 appears to have an additional function as a "heme/oxygen cell sensor", accounted for by the presence of an oxygen-sensing consensus region in the sequence of the gene[84].

As compared with the other two isoforms, HO-3 has low catalytic activity[85]. The differences between the HO isoforms also include the control of their expression, which is probably due to differences in the regulatory elements present in their promoter regions[77].

The expression of HO in human placenta has been studied extensively. The contribution of this enzyme to normal placental function is based on its sub-tissue localization, its enzymatic activity and the ability of the HO-related by-products to exert physiological effects on placental and fetal tissues[86]. Using RT-PCR, the amounts of mRNA encoding both the HO-1 and HO-2 isoforms have been measured in placental tissue. These studies demonstrated an elevated expression of HO-2 as compared to that of HO-1[87-89]. Moreover, the placental expression of both HO-1 and HO-2 increased as gestation advanced[88].

The cytoprotective properties of HO are partly due to the products of its activity, such as CO and biliary pigments. Notably, while the clinical toxicity of CO is clearly recognized, much smaller quantities of CO are remarkably cytoprotective, antiapoptotic, vasorelaxant, and anti-inflammatory[90]. As has already been mentioned, biliary pigments, long regarded to have adverse consequences in hyperbilirubinemic states, are now recognized as anti-inflammatory and antioxidant when present in low concentrations.

Biliverdin-IXα and subsequently bilirubin-IXα are the major biliary pigments in humans. However, small amounts of the other three isomers are also generated depending on the position of the protoporphyrin IX that is cleaved. These include biliverdin-IXγ, biliverdin-IXδ and biliverdin-IXβ, which is the most abundant of these three pigments in humans and other mammals[35].

Biliverdin, which is produced by HO-1 and HO-2 activity, is further biotransformed to bilirubin by biliverdin reductase: mainly biliverdin-IXα reductase (BVRα). This enzyme also functions as a kinase and as a transcription factor in the MAPK signalling cascade[91]. BVRα is expressed in many tissues[92], including the placenta[27]. Bilirubin is conjugated in the liver with glucuronic acid by bilirubin uridine diphosphate-glucuronosyl transferase-1A1 (UGT1A1)[93] prior to being secreted into bile. Owing to the immaturity of the fetal liver, no hepatobiliary elimination of bilirubin occurs, at least at a physiologically relevant rate.

Unconjugated bilirubin concentrations are higher in fetal than in maternal serum[19,26]. Several factors contribute to the existence of this gradient. In the fetus, there is a very active heme catabolism, and hence a high rate of bilirubin production, together with a very low expression of bilirubin uridine diphosphate-glucuronosyl transferase in the liver[94]. Moreover, simple diffusion is not the major route for the placental transfer of biliary pigments[56].

In the presence of reactive oxygen species, bilirubin is oxidized to biliverdin and then converted back into bilirubin by BVRα[95]. Thus the biliverdin-bilirubin tandem acts as an efficient scavenger of reactive oxygen species and inhibits lipid oxidation both *in vitro* and *in vivo*[96,97]. Bilirubin is also an effective antioxidant of peroxynitrite-mediated protein oxidation and inhibits the production of superoxide by blocking the activation of NADPH oxidase[98,99]. Sub-toxic bilirubin concentrations have direct anti-oxidant properties and indirect beneficial effects against cholestasis-induced toxicity during pregnancy, such as the enhanced expression of several elements of the anti-oxidant defence system, i.e. BVRα, SVCT1 and SVCT2, as well as several nuclear receptors sensitive to activation by biliary compounds[100]. This function is mainly dependent on the expression of BVRα, which has been found to be moderately up-regulated in the maternal liver-placenta-fetal liver trio in pregnant rats with surgically induced obstructive cholestasis during the last week of gestation[100]. However, beneficial antioxidant properties are limited to low bilirubin concentrations because at higher levels this pigment can also cause irreversible damage or even death when it is accumulated in the nervous system[101].

It has been suggested that the reduction of biliverdin to bilirubin could have the evolutionary advantage of facilitating the placental excretion of bile pigments by simple diffusion[102]. However, *in vitro*[103] and *in vivo*[104] studies have suggested that under normal physiological circumstances the major pathway for bilirubin placental transfer involves carrier-mediated transport across both poles of the plasma membrane of the human trophoblast[103]. Moreover, at least in rodents, bilirubin does not undergo any major biotransformation during its residence in the placenta[104]. The existence of vectorial properties for transplacental bilirubin transfer are consistent with the moderate increases in serum bilirubin concentrations observed in the fetuses of pregnant rats with marked hyperbilirubinemia due to common bile duct ligation[47]. The mechanism for the placental uptake of fetal biliary pigments is not completely understood. Proteins of the OATP family, in particular human OATP1B1 and OATP1B3, have been reported to confer the ability to take up unconjugated bilirubin when expressed in *Xenopus laevis* oocytes[63]. However, the mRNA of OATP1B1 is almost absent in isolated human trophoblast cells, whereas OATP1B3 is clearly expressed in this epithelium, although at low levels[63].

Inside trophoblast cells, bilirubin is probably partly bound to lipids and proteins such as glutathione-S-transferase[105]. Functional studies have suggested that bilirubin might be exported across the apical pole of the trophoblast *via* an ATP-dependent mechanism[103]. Whether one or several isoforms of MRPs expressed in human[71] and rat[42,68] placenta are involved in this process is not known.

MRP2, and probably MRP1, are also able to perform ATP-dependent transport of bilirubin glucuronides[5]. However, owing to the low UDP-glucuronosyl transferase activity of the fetal liver and the absence of placental biotransformation of unconjugated bilirubin during transplacental transfer[106], MRP2 and MRP1 are not expected to play an important role in bilirubin transfer across the placenta.

It has been shown that biliverdin itself is poorly transferred-without prior reduction to bilirubin - across the guinea pig[26] and rat[106] placenta. However, biliverdin is able to inhibit bilirubin transfer in rat placenta when co-administered through the umbilical artery of *in situ* perfused rat placentas[106]. The transport of biliverdin from the trophoblast toward the mother is very poor and/or that placental biotransformation of biliverdin into bilirubin is very efficient. Part of the endogenous biliverdin produced by the fetus could be transformed into bilirubin by the fetal liver prior to being taken up by the placenta, because the expression of BVRα in fetal liver is even higher than in rat placenta[100].

Among the transporters involved in fetal biliverdin uptake by rat placenta, several OATPs, in particular Oatp1a1, may be involved[106]. Once in the placenta, and prior to being transferred to the mother, biliverdin is extensively converted into bilirubin by BVRα, which is highly expressed in this organ[106]. The small amount of biliverdin that reaches the maternal blood is efficiently taken up, probably in part by Oatp1a1, Oatp1a4 and Oatp1b2, and biotransformed into bilirubin, which joins the fetal bilirubin transferred by the placenta, to be eliminated mainly through secretion into the bile by the maternal liver[106].

PROTECTION AGAINST DRUGS AND TOXINS

Fetal exposure to foreign molecules is partly dependent on the maternal capacity to eliminate such compounds and on the ability of the xenobiotics to cross the placenta. One important characteristic of the placenta is that this organ undergoes continuous development. This implies the existence of changes that must be compatible with the maintenance of a partially permeable epithelial barrier required to provide protection against exposure to potentially harmful substances present in the maternal blood[56,107,108]. Therefore, before any pharmacological interventions, the different stages of pregnancy should be considered, because these will determine both the permeability of the placental barrier and the vulnerability of the conceptus to xenobiotics[109].

Although most drugs administered during pregnancy may cross the placenta to some extent, the magnitude of this depends on the size and structure of the molecule. Diffusional transfer across the placenta for drugs with a molecular weight higher than 500 Da is usually very restricted[110]. Liposolubility and ionization are strong determinants for drug diffusion across the placenta. For instance, several penicillins, in spite of being strong acids, can be efficiently transferred across the human placenta, probably by simple diffusion[111]. Among weak-base drugs, acetaminophen, phenobarbital, phenytoin and clonidine are able to cross the placenta at a high rate, probably by simple diffusion[112]. Nucleoside analogue reverse transcriptase inhibitors (NRTIs) are molecules with low molecular weight and low protein binding, so most of them are also able to cross the placenta by simple diffusion and are concentrated in the amniotic fluid[113].

Carrier-mediated uptake

Although most transporters localized at the plasma membrane of cells and forming part of the placental barrier have specific physiological substrates, some of them also transport structurally similar compounds. In some cases, however, there are no known physiological substrates and only certain xenobiotics have been reported to be transported by them. Moreover, some of the xenobiotics able to cross the placental barrier may have the ability to affect gene expression. This may result in a decrease in the expression of placental transporters, which may affect their ability to accomplish their physiological roles and eventually lead to an enhanced entry of drugs into placental tissue[114].

The placenta expresses some isoforms of monocarboxylate transporters (MCTs)[115]. The primary substrate of MCTs in placenta is lactate, although pyruvate and β-hydroxybutyrate are also transported. Placental MCTs exert a significant influence on the transfer across the maternal-fetal interface of drugs such as valproate, benzoate, salicylates, statins, nateglinide, and foscarnet[114,115].

Equilibrative nucleoside transporters (ENTs) are widely distributed and have broad substrate specificity. There is evidence of the presence of two ENT isoforms in the human placenta: ENT1 (*SLC29A1*) and ENT2 (*SLC29A2*)[116,117]. Moreover, concentrative nucleoside transporters (CNTs), CNT2 (*SLC28A2*) and CNT3 (*SLC28A3*) are also expressed in human placenta. Both ENT1 and ENT2 are able to transport a wide variety of therapeutic agents such as the anticancer drugs cytarabine and gemcitabine and the antiviral drugs zalcitabine (ddC) and zidovudine[117,118], and they therefore probably play a role in fetal exposure to these types of drugs.

Regarding amino acids and monoamines, 17 mammalian transport systems for amino acids have been functionally identified in the human placenta[119,120]. The interaction of xenobiotics with amino acid transport systems in the syncytiotrophoblast may result in a deficit in the transport of amino acids across the placenta. This seems to be the case for cocaine, which readily crosses the placental barrier and enters the fetal circulation. This constitutes a potential cause of adverse effects on the developing fetus in pregnant women consuming this drug[121]. Maternal smoking during pregnancy also decreases the ability of the placenta to efficiently take up amino acids and hence affects the overall transfer of these important metabolites from the maternal to the fetal circulation[121].

Additionally, cocaine may also interact with other placental carriers, such as those involved in monoamine

transport[121]. This may affect serotonin and noradrenaline transport across the apical (maternal-facing) plasma membrane of the trophoblast[122]. Moreover, antidepressants (fluoxetine, paroxetine, sertraline, citalopram, and desipramine) as well as cocaine are inhibitors of monoamine transporters but are not transportable substrates. In contrast, amphetamines are transportable substrates for monoamine transporters, thereby gaining access into the placenta and fetus[122,123].

Many xenobiotics are substrates of OATP isoforms[124], which are expressed in the human placenta[63,66]. These transporters have partially different and overlapping substrate preferences for a wide range of exogenous organic solutes, including gadodexate, ouabain, iloprost, Gd-B 20790, methotrexate, rifampicin, the endothelin receptor antagonist BQ-123, the thrombin inhibitor CRC-220, the opioid receptor agonists D-penicillamine-(2,5)-enkephalin (DPDPE) and deltorphin Ⅱ, the angiotensin-converting enzyme inhibitors enalapril and temocaprilat, the HMG-CoA reductase inhibitor pravastatin, and the antihistamine fexofenadine, in addition to several cytostatic derivatives obtained by coupling bile acid moieties to chlorambucil or cisplatin[125-127]. Some OATP isoforms have also been shown to transport bulky organic cations[124,128]. This suggests that isoforms detected in placenta could serve as a route for the transfer of anions and relatively hydrophilic cationic organic drugs.

Organic cations can be transferred across the placenta using a different route. At least one member of the subfamily of carriers for organic cations (OCTs), namely OCT3, is very abundantly expressed in the human placenta[129,130]. Examples of OCT3 substrates include cimetidine, MPP^+, agmatine, tetraethylammonium, and prazosin[131]. The sodium-dependent carnitine transporter (OCTN2) also belongs to the *SLC22A* family and is expressed in human placenta[132]. OCTN2 transports a variety of organic cations including tetraethylammonium, nicotine, MPP^+, pyrilamine, cimetidine, clonidine, procainamide, quinidine, quinine, and verapamil[133] and certain β-lactam antibiotics of zwitterionic nature[134].

Metabolic barrier

During the first trimester of pregnancy, a broader variety of xenobiotic-metabolizing enzymes are expressed in the placenta as compared to at term[135,136]. However, placental expression of phase I and Ⅱ metabolizing enzymes is moderate and probably more closely involved in the endocrine functions of this organ than in the metabolism of xenobiotics[135]. The placenta expresses several cytochrome P450 enzymes (CYPs) at mRNA levels that increase throughout pregnancy. Although placental CYPs are capable of metabolizing several xenobiotic compounds at term[135,136], only a few of these enzymes are actually functionally active[137,138]. Moreover, the abundance of some of these CYPs has been shown to be affected by exposure to xenobiotics, as occurs in tobacco-smoking pregnant women[138,139]. Other phase I metabolizing enzymes such as aldehyde dehydrogenases (ALDHs) participate in the detoxification of endogenous and exogenous compounds, including ethanol. The presence of this activity in human placenta may be relevant in the toxicity of a number of substances and for the gestational consequences of alcohol consumption[140].

Among phase Ⅱ enzymes, glutathione-S-transferases, epoxide hydrolase, N-acetyltransferases, sulfotransferases, and UDP-glucuronosyl transferases are expressed at moderate levels in the placenta and have been shown to be involved in the detoxification of several xenobiotics[141]. In contrast, drug- and toxin-induced upregulation of biotransforming enzymes can lead to an enhanced production of reactive metabolites able to interact with DNA, resulting in the formation of DNA adducts[142]. This may challenge the normal development of the *conceptus*. Indeed the levels of smoking-related adducts in the placenta have been inversely correlated with offspring birth weight[143].

Export systems

ATP-dependent efflux transporters expressed in the apical membrane of placental syncytiotrophoblasts are very important in limiting the magnitude of drug penetration across the placental barrier, hence reducing fetal drug exposure. The superfamily of ABC proteins includes a large number of members with the ability to translocate a broad variety of substrates across extra- and intracellular membranes. These proteins are involved in many physiological processes, such as sterol homeostasis, immune mechanisms, and the transport of endogenous and xenobiotic substances such as sugars, amino acids, metal ions, peptides and proteins, and a large number of hydrophobic compounds and metabolites. Several members of three families of ABC transporters, ABCB, ABCC and ABCG, known to be involved in multidrug resistance are major candidates for involvement in the placental barrier for drugs[144,145].

The first ABC transporter recognized to play a significant role in the placental barrier was MDR1[145]. MDR1 is abundantly expressed during pregnancy, and in particular in the syncytiotrophoblast[146]. The substrates of MDR1 are usually organic molecules ranging in size from about 200 Da to almost 1900 Da. Most of them are uncharged or weakly basic in nature, but some acidic compounds can also be transported. As a consequence, a large number of drugs from several pharmacotherapeutic groups are recognized as MDR1 substrates. Thus, placental MDR1 may contribute to the protection of the foetus from a wide variety of drugs, including antivirals and anticancer agents[147].

Other major efflux transporters involved in the protection of the developing fetus from exposure to these drugs are members of the MRP subfamily, involved in the transport of conjugates of several drugs and endogenous compounds, have been found in the human placenta. MRP2 is expressed in the syncytiotrophoblast, whereas MRP1 and MRP3 are expressed both in blood vessel endothelia and in the syncytiotrophoblast[71], and MRP5 is expressed in the basal membrane of syncytiotrophoblasts and around fetal vessels[148], where aside from its potential role in drug disposition this transport-

er may mediate the cellular efflux of 3',5'-cyclic nucleotides, cAMP, and cGMP, thus playing an important role in paracrine signal transduction.

BCRP expression in the placenta is possibly tightly controlled during pregnancy by pregnancy-related steroid hormones, growth factors, and cytokines[149]. BCRP transports a broad variety of conjugated or non-conjugated organic anions, but from a physiological point of view it is probably involved in the elimination of endogenous sulphate conjugates[150]. Substantial variations in BCRP expression have been observed in human placenta[151], suggesting that considerable variability could exist in the ability of the placenta to protect the fetus from exposure to drugs, xenobiotics and metabolites. Such variable expression and/or activity has been suggested to be due to genetic polymorphisms in the *BCRP* gene[151].

CONCLUSION

The immaturity of the fetal hepatobiliary system precludes the use of this mechanism of defence against endogenous and xenobiotic compounds during intrauterine life. Consequently, this function is carried out by a complex and efficient combined action of the placenta and the maternal liver. However, when one of these two members of the defensive tandem is impaired the overall function may be compromised, resulting in deleterious effects in the fetus. A better understanding of the molecular mechanisms involved in hepatobiliary excretory function during intrauterine life is needed to recognize the danger the fetus may face, to develop novel pharmacological tools to manipulate the placental transfer of xenobiotics, and to generate new drugs with enhanced or reduced ability to cross the placental barrier.

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Imaging features of solid pseudopapillary tumor of the pancreas on multi-detector row computed tomography

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Abstract

AIM: To retrospectively analyze the imaging features of solid-pseudopapillary tumors (SPTs) of the pancreas on multi-detector row computed tomography (MDCT) and define the imaging findings suggestive of malignant potential.

METHODS: A total of 24 consecutive cases with surgically and pathologically confirmed SPTs of the pancreas underwent preoperative abdominal MDCT studies in our hospital. All axial CT images, CT angiographic images, and coronally and sagittally reformed images were obtained. The images were retrospectively reviewed at interactive picture archiving and communication system workstations.

RESULTS: Of the 24 cases of SPTs, 11 cases (45.8%) occurred in the pancreatic head and seven (29.1%) in the tail. Eighteen were pathologically diagnosed as benign and six as malignant. MDCT diagnosis of SPTs was well correlated with the surgical and pathological results (Kappa = 0.6, $P < 0.05$). The size of SPTs ranged from 3 to 15 cm (mean, 5.8 cm). When the size of the tumor was greater than 6 cm (including 6 cm), the possibilities of vascular (8 *vs* 1) and capsular invasion (9 *vs* 0) increased significantly ($P < 0.05$). Two pathologically benign cases with vascular invasion and disrupted capsule on MDCT presented with local recurrence and hepatic metastases during follow-up about 1 year after the resection of the primary tumors.

CONCLUSION: Vascular and capsular invasion with superimposed spread into the adjacent pancreatic parenchyma and nearby structures in SPTs of the pancreas can be accurately revealed by MDCT preoperatively. These imaging findings are predictive of the malignant potential associated with the aggressive behavior of the tumor, even in the pathologically benign cases.

Key words: Solid pseudopapillary tumor; Pancreas; Multi-detector row computed tomography; Malignant potential; Aggressive behaviors

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INTRODUCTION

Solid pseudopapillary tumors (SPTs) of the pancreas are rare and predominantly occur in young women in the second and third decades of life, with only about 8.3% of all cases reported in males[1,2]. SPT is usually a benign tumor with low-grade malignant potential; however, about 14.7% of cases demonstrate malignant behavior with recurrence and metastasis[3]. In contrast to the ductal adenocarcinoma of the pancreas, complete resection of the tumor could provide a more than 90% cure rate[4,5].

Currently, the imaging modalities such as computed tomography (CT) including multi-detector row computed tomography (MDCT), magnetic resonance

imaging (MRI), and ultrasonography (US) have been used for detection and characterization of pancreatic tumors in clinical practice[6,7]. It is well known that MDCT can provide more accurate delineation of normal and abnormal pancreatic morphology[6]. Furthermore, MDCT can be performed intrinsically for CT angiography (CTA) scanning, and the CTA images can be conveniently obtained by post-processing techniques at the dedicated workstation. However, to the best of our knowledge, there has been no published literature regarding the imaging features implying the malignant potential of the SPTs of the pancreas on MDCT.

This study aimed to retrospectively analyze the imaging features of SPTs of the pancreas on MDCT, and define the imaging findings suggestive of the malignant potential associated with aggressive behavior.

MATERIALS AND METHODS

Patient population

From June 2001 to June 2008, a total of 24 consecutive patients with surgically and pathologically confirmed SPTs of the pancreas, ranging in age from 11 to 64 years (mean 34.27 years; 21 females, three males), underwent preoperative abdominal MDCT studies in our hospital. Abdominal pain or discomfort was the major indication to undergo the imaging studies (87.5%; 21/24). The youngest female patient (11 years old) and a 21-year-old woman both presented with a big mass in the upper abdomen, while the remaining one demonstrated jaundice and elevation of liver enzymes, suggestive of impairment of liver function. The patients have been followed up for 3 mo to 7 years. Institutional review board approval and waiver of informed consent for this retrospective study were obtained.

Imaging techniques

All abdominal MDCTs were performed on a 4-slice or 16-slice multi-detector row CT scanner (LightSpeed QX/I or Lightspeed 16; GE Medical Systems, Milwaukee, WI, USA). All axial CT images were obtained during breath-holding before (non-enhanced), 25 s (arterial phase), 60 s (portal phase), and 90 s (hepatic parenchyma phase) after the initial administration of contrast materials. Contrast-enhanced MDCT was performed after the intravenous injection of iohexol (Omnipaque 300; Amersham, Shanghai, China) at a dose of 2 mL/kg body weight through an antecubital vein using a power injector (LF CT 9000; Liebel-Flarsheim, Cincinnati, OH) at a flow rate of 2.5 to 3.0 mL/s. The major scanning parameters were as follows: beam pitch, 1.5; beam collimation, 4-2.5 mm; gantry rotation time, 0.5-0.8 s; section thickness, 3.75 mm; reconstruction interval, 3.75 mm; and table speed, 15.0 mm/rotation. Axial images were reconstructed with a soft tissue algorithm. The CTA and reformed images were obtained using maximum intensity projection (MIP) or multiplanar volume reformation (MPVR) technique on the workstation. Besides MDCT, 15 cases underwent MRI, and 18 cases underwent US.

Imaging analysis

All images were retrospectively reviewed at interactive picture archiving and communicating system (PACS) workstations by two experienced abdominal radiologists (Wang DB and Chai WM) in consensus. Disputes in readings were resolved through consultation with a third experienced abdominal radiologist (Chen KM). The items included in the imaging analysis were: (1) size, location, and density, as well as the hemodynamics of the tumor; (2) vascular or capsular invasion, invasion of tumor into the adjacent pancreatic parenchyma, and involvement of nearby vessels as well as metastases in lymph nodes and other places in the upper abdomen. All the reviewers were unaware of the pathological nature regarding the benignity and malignancy for each case. Based on the imaging findings of the SPTs of the pancreas on MDCT, each case was given a diagnosis of either benign or malignant SPT.

Pathological studies

Histopathological and immunohistochemical studies were performed in all the cases. The diagnosis of SPTs by MDCT was compared with the pathological results.

Statistical analysis

The Fisher's exact test and Kappa test were introduced for comparison of imaging diagnosis and pathological results. A P value less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed using the SPSS computer software (Version 13.0, Chicago, IL, USA).

RESULTS

Surgical and pathological results

Of the 24 cases, 18 were diagnosed as benign SPTs while the other six as malignant on pathological study. The size of SPTs ranged from 3 to 15 cm (mean 5.8 cm). One of the malignant SPTs metastasized to the liver unifocally and the regional lymph nodes. The metastatic lesions were also resected simultaneously during the operation of the primary tumor in the pancreatic head. There were another two cases of malignant SPT with regional lymph node metastasis found in surgery. The involvement of nearby vessels (n = 8, including two benign cases), adjacent organs including spleen (n = 1), duodenum (n = 1), and stomach (n = 1), and abutting pancreatic parenchyma (n = 6), as well as the peripancreatic fat (n = 6), was revealed during the operations (Table 1). One of them had a thrombus in the portal vein. Complete resection was performed for each case in this group.

Imaging features of SPTs on MDCT compared with pathological results

Table 1 summarizes the imaging features of SPTs on MDCT in comparison with pathological outcome. Of the 24 cases of SPTs, 11 (45.8%) occurred in the pancreatic head and seven (29.1%) in the tail (Table 1). The calcifications were identified in five cases (three

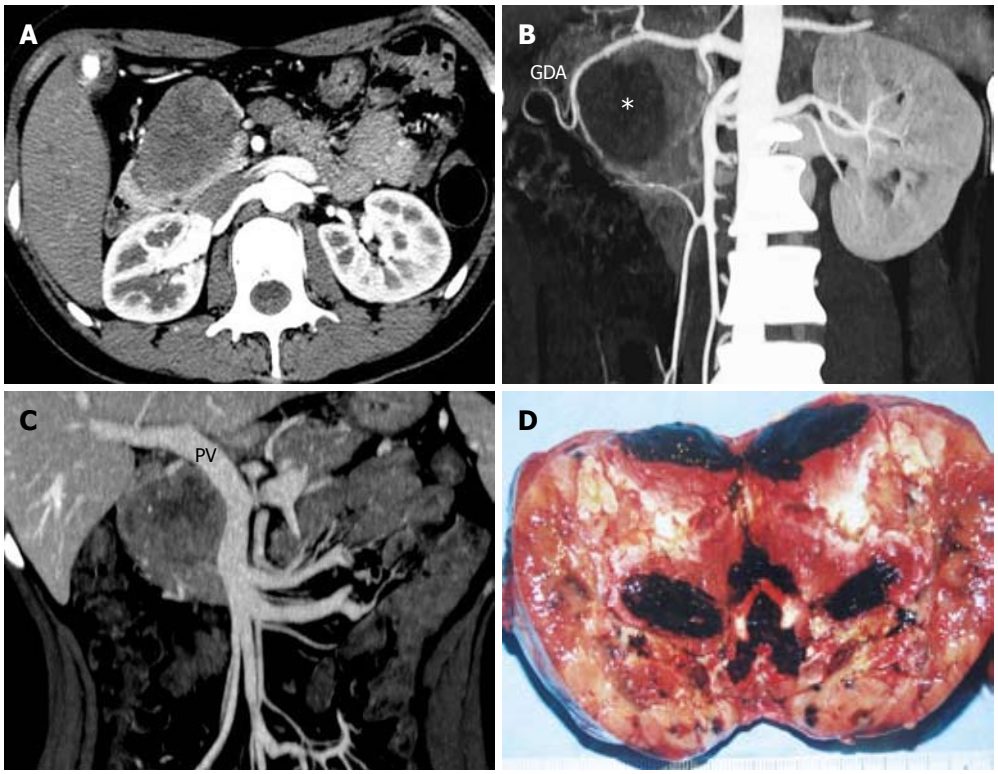

Figure 1 Pathologically confirmed benign SPT in a 24-year-old woman with abdominal discomfort for 1 year. A: Axial image at arterial phase revealed a low-attenuation mass in the pancreatic head. The surrounding vessels were displaced; B: The MIP CTA image identified that the tumor (*) displaced the gastroduodenal artery (GDA) without infiltration; C: The MPVR image demonstrated that tumor compressed the portal vein (PV) with a smooth border. There was no evidence suggesting invasion; D: Hemorrhagic and cystic areas (dark areas) were detected in the gross specimen. The capsule was intact.

benign, two malignant). On MDCT, the tumors were divided into three types according to the density: cystic, solid, and solid-cystic (mixed) (Figure 1). The mixed type comprised 58.3% (14/24) of the cases of SPT. Six malignant and three benign cases were identified with a disrupted capsule, whereas the other cases had an intact and smooth capsule. In this group, 50% (12/24) of the SPTs demonstrated moderate enhancement of the tumors after administration of contrast agents. Nine of the 12 cases were benign. However, among the six cases with marked enhancement, three were malignant. Peripheral and persistent enhancement in solid components occurred in 95.8% (23/24) of the cases. The MDCT identified all surgically and pathologically confirmed tumoral invasion into the adjacent structures and organs (spleen, duodenum and stomach) and metastases (lymph nodes, $n = 3$; liver metastasis, $n = 1$) in six malignant cases (Table 1). However, the adjacent pancreatic invasion ($n = 3$), pancreatic duct dilatation ($n = 1$), peripancreatic fat invasion ($n = 1$), and nearby vascular involvement ($n = 2$) in benign cases were also depicted on MDCT. The subsequent pancreaticoduodenectomy operation was performed on four of these cases, and pathological study revealed the benign nature in spite of encasement of the nearby portal vein. Therefore, eight cases were diagnosed as malignant SPTs by MDCT, including six categorized as malignant by pathology (75%; 6/8). Finally, MDCT diagnosis of SPTs was well correlated with the surgical and pathological results (Kappa = 0.6, $P < 0.05$) (Table 2).

When the size of the tumor was $\geqslant$ 6 cm, the possibilities of vascular involvement (8 *vs* 1) and capsular invasion (9 *vs* 0) increased significantly ($P < 0.05$) (Tables 1 and 3). The disrupted capsule detected on MDCT was attributed to the tumoral invasion into adjacent pancreatic parenchyma or peripancreatic structures by pathologically confirmed SPT in this group (Figure 2). Table 4 demonstrates that the portal vein, superior mesenteric vein, and splenic vein were vulnerable to being involved. Two pathologically benign cases with vascular involvement and disrupted capsule on MDCT presented with local recurrence and hepatic metastases during follow-up, about 1 year after resection of the primary tumors (Figure 3).

DISCUSSION

SPT of the pancreas is rare, mainly occurring in young women. It was first described by Frantz in 1959[8] and the tumor was named the Frantz tumor after the author. Over time, it has been designated with various names such as: solid and papillary tumor[9]; papillary cystic tumor[10]; solid-cystic tumor[11]; and solid and papillary epithelial neoplasm[12]. However, these names do not exactly reflect what is present either at the microscopic or macroscopic level. WHO in 1996 proposed the name 'solid-pseudopapillary tumor', which can depict two major histological features in the tumor: solid and papillary components[13]. In fact, SPTs have been subdivided by the WHO classification into: solid-

Table 1 Imaging features of SPTs on MDCT in comparison with surgical and pathological results *n* (%)

Imaging features on MDCT	Surgical and pathological results	
	Benign (*n* = 18)	Malignant or potentially malignant (*n* = 6)
Location		
Head	8 (33.3)	3 (12.5)
Neck	1 (4.2)	0
Body	2 (8.3)	1 (4.2)
Tail	5 (20.8)	2 (8.3)
Body-neck	1 (4.2)	0
Body-tail	1 (4.2)	0
Size		
< 6 cm	10 (41.7)	1 (4.2)
⩾ 6 cm	8 (33.3)	5 (20.8)
Density		
Cystic	5 (20.8)	0
Solid	3 (12.5)	2 (8.3)
Solid-cystic	10 (41.7)	4 (16.6)
With calcifications	3 (12.5)	2 (8.3)
Capsule		
Intact	15 (62.5)	0
Un-intact	3 (12.5)	6 (25)
Enhancement		
Mild	6 (25)	0
Moderate	9 (37.5)	3 (12.5)
Marked	3 (12.5)	3 (12.5)
Enhancing pattern and hemodynamics		
Peripheral and persistent enhancement	17 (70.8)	6 (25)
Central and persistent enhancement	1 (4.2)	0
Pancreatic or peripancreatic invasion		
Adjacent pancreatic invasion	3 (12.5)	6 (25)
Pancreatic duct dilatation	1 (4.2)	1 (4.2)
Peripancreatic fat invasion	1 (4.2)	6 (25)
Nearby vessels involvement	2 (8.4)	6 (25)
Direct invasion into adjacent organs	0	3 (12.5)
Regional lymph nodes metastasis	0	3 (12.5)
Liver metastasis	0	1 (4.2)

Table 2 Comparison of MDCT diagnosis and pathologic results in SPTs

MDCT	Pathologic results		Total
	Malignant or malignant potential	Benign	
Malignant	5	3	8
Benign	1	15	16
Total	6	18	24

Kappa = 0.6, $P < 0.05$.

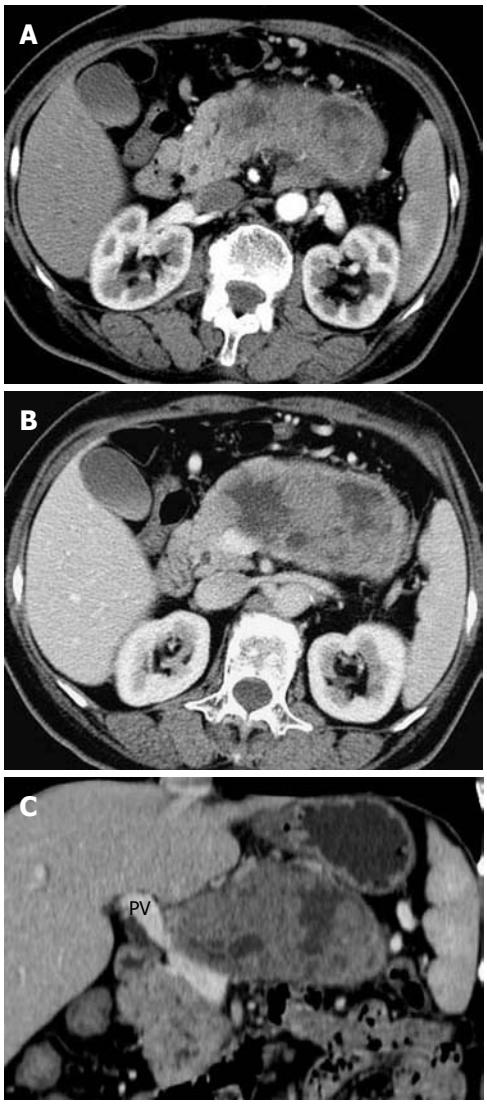

Figure 2 Malignant SPT in a 35-year-old woman with abdominal pain and mass for 6 mo. A: A mass measuring 13 x 5.5 cm was detected on axial MDCT imaging in the pancreatic body. The mass was markedly enhanced and superimposed by abnormal enhancement of tiny vessels at the arterial phase. The interface between the tumor and the adjacent pancreatic parenchyma was blurred in that the tumor apparently infiltrated the surrounding pancreas; B: At the portal venous phase, the axial image demonstrated the heterogeneity of cystic and solid components inside the tumor. The peritumoral capsule was not smooth, which was consistent with capsular invasion. The portal vein was deformed by tumoral invasion; C: Coronal MPVR image showed the tumoral invasion resulting in the narrowing and irregularity of portal vein (PV).

Table 3 Correlation between tumor size and aggressive behaviors in SPTs

Tumor size	Vascular involvement		Metastasis		Capsular invasion	
	Positive	Negative	Positive	Negative	Positive	Negative
< 6 cm	1	10	0	11	0	6
⩾ 6 cm	8	5	[1]3	10	9	4

Fisher's exact test: $P = 0.013$ for vascular involvement; $P = 0.001$ for capsular invasion. [1]2 cases with lymph node metastasis and 1 case with lymph node metastasis and hepatic metastasis.

papillary neoplasm with borderline malignant potential, and solid-papillary carcinoma[14]. To date, more than 700 cases have been reported in the English literature[15]. About 15% are known to present with metastasis or recurrence[3]. However, based on the conventional histopathology, it has been difficult to establish the criteria that are suggestive of aggressive behavior including recurrence and metastasis[14,16-17].

CT is the imaging modality of choice for detection and characterization of SPTs of the pancreas, while the MRI can be more accurate in differentiating the cystic or solid component inside the tumor[7]. If MRI reveals an encapsulated mass lesion with solid and cystic component as well as hemorrhage without obvious internal septations, SPT of the pancreas should be suspected[7]. According to Yu[7], the MRI

Table 4 Vascular invasion on MDCT in comparison with surgical and pathological results in six malignant and two benign SPT cases

MDCT	Surgical and pathological results							
	PV (n = 5[1])	SV (n = 5)	SMV (n = 5)	CA (n = 2)	CHA (n = 3)	PHA (n = 3)	SA (n = 3)	SMA (n = 3)
Axial images	3	1	2	0	3	3	1	0
CTA MIP images	5	3	5	1	3	3	4[2]	3
MPVR images	5	3	4	1	3	3	3	3
A+C+M	5	5	5	2	3	3	3	3

[1]indicating three malignant and two benign SPTs; [2]one of four cases was overestimated; CTA: Computed tomography angiography; MIP: Maxiumum intensity projection; MPVR: Multiplanar volume reformation; A+C+M: Combination of axial images and CTA MIP and MPVR images. PV: Portal vein; SV: Splenic vein; SMV: Superior mesenteric vein; CA: Celiac artery; CHA: Common hepatic artery; PHA: Proper hepatic artery; SA: Splenic artery; SMA: Superior mesenteric artery.

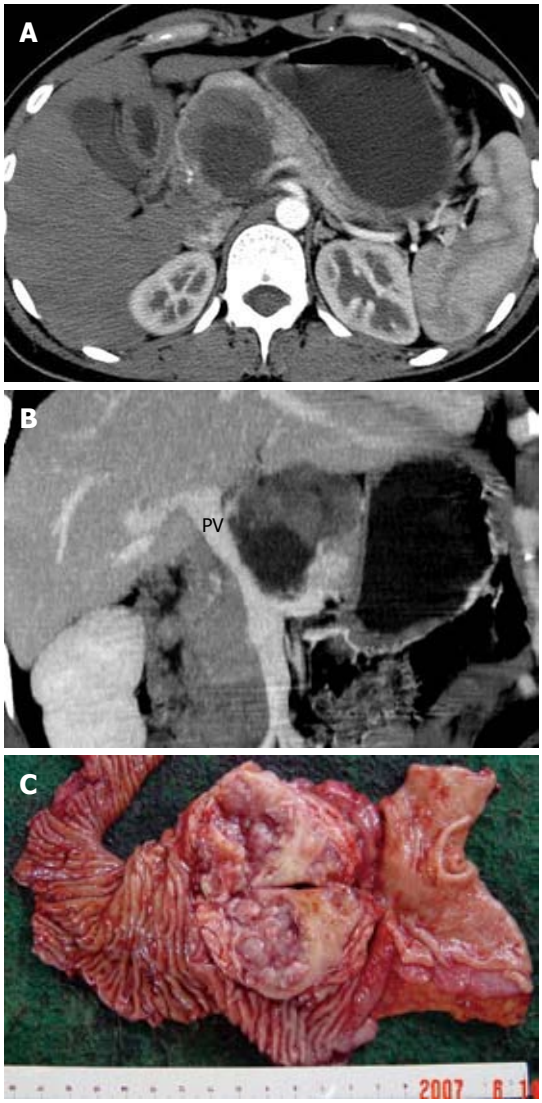

Figure 3 Pathologically confirmed benign SPT in a 30-year-old woman with abdominal pain for about 3 mo. A: A heterogeneously low-attenuation mass was identified in the pancreatic head on the axial MDCT image at the arterial phase. The capsule was irregular and not attached to the adjacent, pancreas implying invasion outside the mass. The peripheral portion of the mass close to the duodenum was enhanced significantly and the duodenum was infiltrated; B: MPVR image revealed the irregularity and narrowing of the portal vein (PV) indicating vascular invasion; C: Invasion into the duodenum was revealed in the gross specimen of the tumor.

findings of SPTs were well correlated with the pathological patterns. Fine needle aspiration biopsy guided by endoscopic ultrasound can help distinguish SPTs from other pancreatic lesions, since the masses are commonly localized in the pancreatic head, thus playing an important role in preoperative planning[18]. Therefore, the imaging modalities are very useful in preoperative assessment of this disease and could provide strong evidence for treatment protocol planning. It is well known that MDCT can provide more accurate delineation of normal and abnormal pancreatic morphology[6]. Furthermore, MDCT can be performed intrinsically for CTA scanning, and the CTA images can be conveniently obtained by post-processing techniques at the dedicated workstation, meanwhile, the coronal and sagittal images can be reformed using axial source images. Thus, axial CT and CTA images can be simultaneously obtained during a single scanning.

The SPT is characterized with imaging features of a well-circumscribed mass, which is surrounded by a clear-depicted peritumoral capsule, and vulnerable to hemorrhage and cystic changes, resulting in the heterogeneously cystic central component and solid periphery. The overview of the clinicopathological characteristics of the SPTs in our series (Table 1) is almost identical to the data reported in the literature[16]. Our MDCT images demonstrated that peripheral and persistent enhancement in solid components occurred in 95.8% (23/24) of all cases. As is well documented in the literature[19], the enhancement of SPTs was not significant in the adjacent pancreatic parenchyma. However, 50% (3/6) of cases in this group with marked enhancement turned out to be malignant. It seems that the hypervascular feature on MDCT could imply an aggressive nature, since half of the malignant cases were categorized as having marked enhancement. Further study is needed to confirm this.

The accuracy of MDCT diagnosis of SPTs was well correlated with the surgical and pathological results (Kappa = 0.6, $P < 0.05$) (Table 2). As a matter of fact, some of the pathologically benign cases with local invasion detected by MDCT in this group might turn out to be malignant during follow-up, as was reported in two cases herein. Although the axial and coronal reformed images, as well as CTA images, could reveal the aggressive behavior of SPTs, the combination of all these images seems to add value to the assessment of the status of tumoral invasion into the adjacent major

vasculature (Table 4). However, statistical analysis was not carried out for the capability of identification of vascular invasion by different images, since the limited number of cases included would bias the results.

In this group, when the mass was more than 6 cm (including 6 cm) in diameter, the tumor was more vulnerable to having aggressive behavior such as metastasis and recurrence. The mean mass size in our series was 5.8 cm. When we set up the threshold of tumor size at 6 cm, the nearby vascular involvement (8 *vs* 1) and peritumoral capsular invasion (9 *vs* 0) were more common in SPTs bigger than 6 cm (including 6 cm), which indicated the biologically aggressive nature revealed by MDCT ($P < 0.05$, Table 3). Interestingly, if the mass was smaller than 6 cm, there was no case categorized as having capsular invasion in this group, which may be wrong if the same size threshold were applied to other groups, since different imaging modalities were employed. As a result of invasion beyond the capsule, invasion into the adjacent pancreatic parenchyma was identified in nine cases, including six malignant cases. Two of the three cases in this settings regarded as benign pathologically had aggressive behavior of vascular involvement and capsular invasion on MDCT, and subsequently presented with local recurrence and hepatic metastasis during follow-up after surgery of the primary tumor. In this context, the findings on MDCT could predict the possible aggressive nature of SPTs of the pancreas, based on our data. Complete surgical resection can be proposed for SPTs and the prognosis is supposed to be excellent[15]. Up to the time of writing, no patient died during the follow-up in our group.

In conclusion, based on our SPT series, local invasion, including vascular and capsular invasion with superimposed spread into the adjacent pancreatic parenchyma and peripancreatic structures and organs, can be accurately depicted by MDCT preoperatively. The findings suggestive of malignancy on MDCT were well correlated with aggressive behavior of the tumor, even in the pathologically benign cases. Therefore, the value of imaging features implying aggressive behavior of SPTs needs to be emphasized; it seems that the imaging findings are predictive of the malignant potential associated with aggressive nature, and are probably beneficial to the patient's surgical protocol planning.

COMMENTS

Background

The solid-pseudopapillary tumor (SPT) of the pancreas is rare, mainly occurring in young women. To date, more than 700 cases have been reported in the English literature. About 15% are known to present with metastasis or recurrence. However, based on conventional histopathology, it has been difficult to establish the criteria that are suggestive of the aggressive behavior including recurrence and metastasis.

Research frontiers

No pathological factors including mitotic rate, nuclear pleomorphism and vascular invasion were found to correlate with the prognosis of SPT. Also, the histopathological features suggestive of malignant potential were non-specific. Aggressive behavior may not be completely excluded, even in the absence of pathological features suggesting malignant potential. CT is the imaging modality of choice for detection and characterization of SPTs of the pancreas while MRI can be more accurate in differentiating the cystic from solid component inside the tumor.

Innovations and breakthroughs

To the best of our knowledge, there is no published literature regarding the imaging features implying the malignant potential of SPTs of the pancreas on multi-detector row computed tomography (MDCT). In our series, when the size of the tumor was greater than 6 cm (including 6 cm), the possibilities of vascular (8 *vs* 1) and capsular invasion (9 *vs* 0) increased significantly ($P < 0.05$). Two pathologically benign cases with vascular invasion and disrupted capsule on MDCT presented with local recurrence and hepatic metastases during follow-up, about 1 year after resection of the primary tumors. Vascular and capsular invasion with superimposed spread into the adjacent pancreatic parenchyma and nearby structures in SPTs of the pancreas could be accurately revealed by MDCT preoperatively. These imaging findings could be predictive of the malignant potential associated with aggressive behavior of the tumor, even in the pathologically benign cases.

Applications

The findings suggestive of the malignancy on MDCT are well correlated with aggressive behavior of the tumor, even in the pathologically benign cases. Therefore, the value of imaging features implying aggressive behavior of SPTs needs to be emphasized. It seems that the imaging findings are predictive of the malignant potential associated with aggressive nature and are beneficial to the patient's surgical protocol planning.

Terminology

"Solid pseudopapillary": Encompasses the two most conspicuous histological features, which are also depicted macroscopically, the cystic center and the solid periphery of the mass. "MDCT": Multiple detectors applied to CT. This modality can improve the scanning speed and spatial resolution dramatically. Furthermore, MDCT can be performed intrinsically for CT angiography scanning. "Aggressive behavior": The tumor with aggressive behavior means that it has local invasion, local recurrence, or metastasis.

Peer review

This is a retrospective study of the radiological features of SPT upon MDCT in 24 consecutive cases. Radiological changes have been correlated with operative and pathological findings. These imaging findings could be predictive of the malignant potential associated with aggressive behavior of the tumor, even in pathologically benign cases.

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ORIGINAL ARTICLES

Detachment of esophageal carcinoma cells from extracellular matrix causes relocalization of death receptor 5 and apoptosis

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Abstract

AIM: To investigate the effect of detachment of esophageal cancer cells from extracellular matrix on the localization of death receptor 5 (DR5) and apoptosis.

METHODS: Anchorage-dependent EC9706 cells of esophageal squamous cell carcinoma were pretreated or not treated with brefeldin A. Detached cells were harvested by ethylenediaminetetraacetic acid digestion. Expression and localization of DR5 in these cells were determined by immunocytochemical and immunofluorescence assays, as well as flow cytometry analysis. Apoptosis of EC9706 cells was detected by flow cytometry after stained with fluorescein isothiocyanate-labeled annexin V/propidium iodide. Activation of caspase 8 was detected by Western blot analysis.

RESULTS: Immunocytochemical assay indicated that DR5 was predominantly perinuclear in adherent cells but was mainly localized in cell membrane in detached cells. In addition, immunofluorescence assay also confirmed the above-mentioned results, and further demonstrated that DR5 was present in the form of coarse granules in detached cells, but in the form of fine granules in adherent cells. Cytometry analysis revealed higher levels of DR5 expression on the surfaces of brefeldin-A-untreated cells than on the surfaces of brefeldin-A-treated cells, but brefeldin A treatment did not affect the total DR5 expression levels. Moreover, nocodazole did not influence the extracelluar DR5 expression levels in EC9706 cells. Apoptosis assay revealed that detached cells were more sensitive to DR5 antibody-induced apoptosis than adherent cells. Western blotting showed that caspase 8 was activated in temporarily detached cells 4 h earlier than in adherent cells.

CONCLUSION: Progress from adhesion to detachment of EC9706 cells causes DR5 relocalization, and promotes cytoplasmic translocation of DR5 to cell surfaces *via* a Golgi-dependent pathway. Moreover, it might also result in DR5 aggregation to render apoptosis of detached cells.

Key words: Translocation of death receptor 5; Cell detachment; Esophageal carcinoma; Anoikis; Apoptosis

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Liu GC, Zhang J, Liu SG, Gao R, Long ZF, Tao K, Ma YF. Detachment of esophageal carcinoma cells from extracellular matrix causes relocalization of death receptor 5 and apoptosis. *World J Gastroenterol* 2009; 15(7): 836-844 Available from: URL: http://www.wjgnet.com/1007-9327/15/836.asp DOI: http://dx.doi.org/10.3748/wjg.15.836

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand

(TRAIL) was identified in 1995 based on its sequence homology to FasL/Apo1L and TNF[1]. TRAIL is a type Ⅱ membrane protein and can be cleaved from the cell surface to form a soluble ligand. Both membrane-bound and soluble TRAIL can rapidly induce apoptosis in a variety of tumor cells and transformed cells, with minimal adverse effects on normal cells[1,2]. In addition, it was reported that recombinant human TRAIL (rhTRAIL), in combination with chemotherapy or radiotherapy, has synergistic effects on several types of human cancer and can overcome resistance to both chemotherapy and radiotherapy[2]. Due to its highly selective tumoricidal activity, TRAIL is a promising agent for cancer therapy and mediates apoptotic effects by binding to its agonistic death receptors as a homotrimer. Several TRAIL receptors have been discovered to date, including TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4. TRAIL-R1 and TRAIL-R2, which are death receptors, commonly known as DR4 and DR5, respectively, transduce TRAIL-mediated death signals to the intracellular apoptotic machinery. However, TRAIL-R3 and TRAIL-R4 are designated as anti-apoptotic decoy receptors that antagonize TRAIL-induced apoptosis[2]. It was reported that DR5 is probably the main TRAIL death receptor[2,3] because it exhibits a considerably higher affinity for TRAIL than DR4 in physiological conditions (37℃)[4], and may play a more prominent role than DR4 in mediating apoptotic signals emanating from TRAIL in cells expressing both death receptors[5]. Thus, DR5 is a good potential target for anti-tumor therapy.

Since TRAIL and agonistic antibodies against DR5 activate membrane-bound DR5 to trigger apoptosis, cellular localization sites of DR5 directly affect TRAIL-induced apoptosis[2,3]. Therefore, it is very important to elucidate the cellular localization of DR5 and the mechanisms underlying the regulation of DR5 expression and transport. Cellular localization of DR5 has been partially determined. There are numerous DR5s in cytoplasm and a few on the cell membrane of human melanoma cells, but DR5 is not present in nuclei[6]. Recently, Laguinge *et al*[7] reported that DR5 expression is increased in detached human colorectal carcinoma cells and DR5 mediates anoikis through a caspase 8-dependent pathway. In the development and progress of malignant tumors, as detachment from primary tumor is an obligatory step in metastasis, tumor cells must detach from their substrates at a distant site to create metastasis. Furthermore, high levels of TRAIL have been reported in tumor-infiltrating lymphocytes of cancer patients, thus high levels of DR5 in tumor cells will decrease the survival and metastasis of detached cells. Therefore, low levels of DR5 are frequently expressed in metastasized cells[8,9]. To date, the mechanisms underlying the expression and translocation of DR5 are still unclear.

In the present study, we investigated the expression, relocalization, and translocation of DR5 in esophageal cancer cells during the process of cell detachment. Our data suggest that detachment of EC9706 cells from extracellular matrix cause DR5 relocalization, promotes cytoplasmic translocation of DR5 to cell surfaces *via* a Golgi-dependent pathway, and might also result in DR5 aggregation. Our findings provide new insights into the mechanisms underlying the regulation of intracellular localization and translocation of DR5 and also indicate the potential clinical applications of DR5 antibodies and TRAIL in anticancer therapy.

MATERIALS AND METHODS

Materials

Neutralizing monoclonal antibody 366 EC and functional monoclonal antibody mDRA-6 with apoptotic activity against DR5 were prepared as previously described[5,10]. Rabbit anti-mouse SP kit and DAB kit were from Zymed Laboratories (San Francisco, CA, USA). Endogenous peroxidase blocking kit was obtained from Vector Laboratories (Burlingame, CA, USA). Goat anti-human caspase-8 antibody was from R&D systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1, horseradish peroxidase (HRP)-conjugated goat anti-mouse and rabbit anti-goat IgG1, and ECL plus Western blotting detection system were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Annexin V-FITC/PI apoptosis detection kit was from BD Pharmingen (San Diego, CA, USA). Polyvinylidene difluoride membrane was from Millipore Corp (Bedford, MA, USA). Fetal bovine serum was from Tianjin Haoyang Biological Manufacture Co., Ltd (Tianjin, China). RPMI-1640 medium, trypsin, and ethylenediaminetetraacetic acid (EDTA) were from Gibco BRL (Gaithersburg, MD, USA). Brefeldin A and nocodazole were from Sigma (St. Louis, MO, USA). Other chemicals used were of analytical reagent grade.

Cell culture

Esophageal cancer cell line EC9706 was obtained from Cancer Institute, Chinese Academy of Medical Sciences (Beijing, China). EC9706 cells were cultured in RPMI-1640 medium supplemented with 100 mL/L fetal bovine serum, 50 IU/mL penicillin, and 50 mg/L gentamycin at 37℃ under an atmosphere containing 50 mL/L CO_2. The medium was changed every 2 d until 80%-90% confluence was achieved. The cells were harvested by 0.3 g/L trypsin-EDTA digestion for subculture or to prepare a single-cell suspension for subsequent experiments.

Detection of DR5 expression by flow cytometry

To detect the cell-surface expression of DR5, EC9706 cells (10^5/well) were seeded in a 24-well plate and incubated for 10 h at 37℃ under an atmosphere containing 50 mL/L CO_2. Adherent cells were incubated in a medium containing brefeldin A at a final concentration of 10 mg/L, or only in the culture medium, for 30 min, and then trypsinized to obtain a single-cell suspension for subsequent experiments. The adherent cells were incubated in the medium containing nocodazole at a final concentration of 10 mg/L, or only

in the culture medium, for 8 h, followed by trypsinization to obtain a single-cell suspension. The cells were washed with a staining buffer (Hank's solution into which 1 g/L BSA and 1 g/L NaN_3 were added). One portion of the cells was used for extracellular staining. These cells were stained on ice with 4 mg/L 366EC antibody in 100 μL of staining buffer for 40 min, washed twice with PBS, and then incubated on ice with FITC-IgG for 40 min, avoiding direct light exposure. After two final washings with PBS, the cells were fixed in a 10 g/L paraformaldehyde (PFA) solution and finally resuspended in 500 μL PBS. Flow cytometry was performed using a FACSCalibur cytometer (BectonDickinson, San Jose, CA, USA). A minimum of 1×10^4 cells per sample was interrogated and the data were analyzed using the CellQuest software (BectonDickinson). The negative control sample used for flow cytometry experiments was treated with normal mouse serum instead of 366EC antibody. All experiments were repeated at least three times.

In order to measure DR5 intracellular protein levels as well as extracellular expression, the remaining cells were permeabilized by methanol and 1 g/L saponin after fixation using 10 g/L PFA. The cells were stained following the same procedure as the extracellular staining except for the lack of a final fixation step in 10 g/L PFA. Total DR5 expression level was analyzed by flow cytometry as described above.

Intracellular localization of DR5

For immunocytochemical staining, the EC9706 cells were cultured as a monolayer in six-well dishes containing sterilized cover-slips at 37℃ for 12 h. The adherent cells were treated with brefeldin A at a final concentration of 10 mg/L, or with culture medium only, for 30 min. The adherent cells plated in six-well dishes were digested by EDTA buffer to obtain a single-cell suspension. After two final washings with PBS, the detached cells were spun on coverslips. After treatment, all cells on the coverslips were fixed in cold methnol: water (1:1) for 10 min and dried for 5 min at room temperature. Endogenous peroxidase was blocked with 3 mL/L H_2O_2 in a PBS solution for 30 min. The coverslips were then incubated with avidin and biotin blocking solutions, pre-incubated with 50 mL/L normal mouse serum and 10 g/L bovine serum albumin (BSA) in PBS for 15 min, and then incubated with primary antibodies (4 mg/L 366EC) at 37℃ for 1 h. After washing with PBS, the coverslips were incubated with biotinylated goat-antimouse antibody at room temperature for 40 min, and then with streptavidin-peroxidase at room temperature for 15 min. Peroxidase activity was observed by incubating the slides for 3 min in a DAB solution. After washed several times, the coverslips were counterstained with hematoxylin (or not counterstained), dehydrated with ethanol, rinsed in xylene, and then mounted with gum for microscopic examination and photography. The negative control sample was treated with normal mouse serum instead of 366EC antibody.

For immunofluorescence staining, the cells were treated as described above. After the cells were incubated with primary antibody, secondary antibody FITC-IgG diluted at 1:200 was added, and incubated at 4℃ in the dark and at room temperature for 1 h, respectively. The staining specificity was analyzed under an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Oberkochen, Germany) using a 40 × objective lens. Image capture was performed with a Spot RT color CCD camera and the Spot RT software (Diagnostic Instruments, Sterling Heights, MI, USA). All experiments were repeated at least three times. The negative control sample was treated with non-immune normal rabbit IgGs instead of primary antibodies.

Analysis of apoptosis

Apoptosis was evaluated using an Annexin V-FITC apoptosis detection kit. Briefly, EC9706 cells (10^5/well) were seeded in a 24-well tissue culture plate and incubated for 10 h. The cells were randomly divided into adhesion group, detachment group, and negative control group. Cells in the adhesion group were treated with 2 μg/mL mDRA-6 antibody for 12 h, cells in the detachment group were trypsinized to prepare a single-cell suspension (detached cells) and then detached cells were treated with 2 mg/L mDRA-6 antibody for 12 h, and cells in the negative control group were incubated in a medium without mDRA-6 antibody for 12 h. Subsequently, floating and adherent cells in the medium were collected and centrifuged at 1800 r/min for 6 min at 4℃. Cell pellets were washed twice with cold PBS and then resuspended in a binding buffer at a concentration of 1×10^6 cells/mL, and 100-μL aliquots of this cell suspension (1×10^5 cells) were then transferred to a 5-mL culture tube. Using an Annexin V-FITC apoptosis detection kit, the cells were stained with AnnexinV-FITC and propidium iodide (PI) according to the manufacturer's instructions. Five microliters of annexin V-FITC and 5 μL of PI were added to each 100-μL aliquot of the above-mentioned cell suspension, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. Each sample, to which 400 μL of 1 × binding buffer was added, was analyzed using a FACSCalibur cytometer within 1 h. A minimum of 1×10^4 cells was detected in each flow cytometry sample and the data were analyzed using the CellQuest software.

Western blot analysis

To assess the caspase-8 activation, after required treatments, cells (3×10^6) were washed once with PBS and lysed in the sample buffer (200 μL) for SDS-PAGE and immediately boiled for 4 min. Each sample was subjected to 15 % SDS-PAGE, and proteins separated on the gel were subsequently electrotransferred onto a polyvinylidene difluoride membrane which was blocked with 50 g/L non-fat dry milk in TBS-T (20 mmol/L Tris-HCl at pH 7.4, 8 g/L NaCl, and 1 mL/L Tween 20) for 2 h at room temperature. The membrane was then incubated with goat anti-human caspase-8 antibody in TBS-T containing 50 g/L non-fat dry milk at 4℃ overnight, washed three times with TBS-T and probed

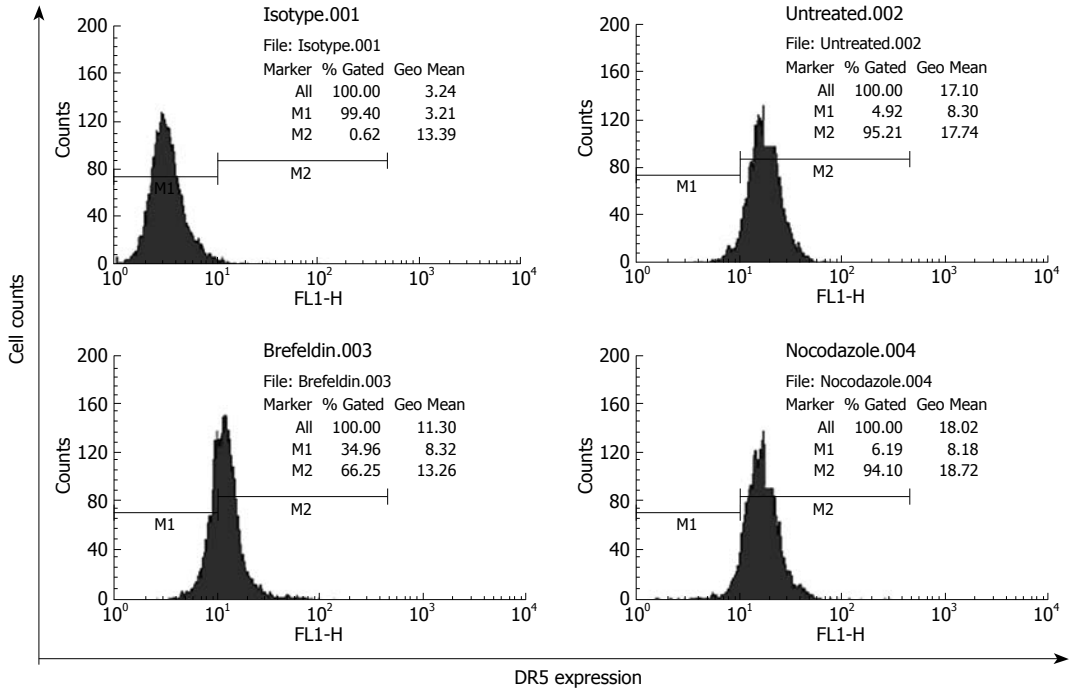

Figure 1 Analysis of DR5 extracellular expression in EC9706 cells by flow cytometry. Isotype.001: Isotype control; Untreated.002: Extracellular expression of DR5 in the cells pretreated without brefeldin A; Brefeldin.003: Extracellular expression of DR5 in the cells pretreated with brefeldin A; Nocodazole.004: Extracellular expression of DR5 in the cells pretreated with nocodazole for 8 h and without brefeldin A.

with peroxidase-conjugated rabbit anti-goat antibody at room temperature for 2 h. After washing four times with TBS-T, the protein was observed using the ECL Plus Western blotting detection system according to its manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SAS® 6.12 (SAS Institute, Cary, NC, USA). All values were expressed as mean ± SD. The F test was used to compare pairs of means. $P < 0.05$ was considered statistically significant.

RESULTS

DR5 expression in EC9706 cells

In the progress of malignant tumors, detachment is very important to the metastasis of tumor cells and tumor cells must detach from their substrates frequently at a distant site to create metastasis. As the expression level of DR5 receptors on cell surfaces directly affects TRAIL-induced apoptosis, it is thus essential to investigate the relation between DR5 expression on cell surfaces and cell detachment. To determine DR5 expression on the surfaces of detached cells, detached cells were harvested after pretreatment with or without brefeldin A, which can specifically disrupt the functions of Golgi apparatus[11] and block proteins trafficking to cell membranes. The detached cells labeled with FITC-IgG were detected by flow cytometry. The DR5 expression rate on cell surfaces was 96.21% in brefeldin A-untreated EC9706 cells. However, it was decreased to 66.25% ($P < 0.01$) and the expression intensity was decreased from 17.34 to 13.26 ($P < 0.05$) in brefeldin-A-treated cells (Figure 1), suggesting that transformation of EC9706 cells from a spreading adherent to a detached status is accompanied with translocation of DR5 to the cell surface.

Brefeldin A treatment did not change the total DR5 expression, indicating that detachment does not influence DR5 synthesis, and increased DR5 expression on the cell surface could not be simply explained by DR5 protein synthesis. Since cytoplasmic DR5 is mainly localized within the Golgi apparatus[6] and brefeldin A can specifically inhibit the functions of the Golgi network[11], the present results indicate that Golgi network is involved in DR5 translocation from the cell interior to the plasma membrane, and DR5 shuttles to the cell surface in a classic protein secretory route. It has been reported that DR5 translocation from the Golgi to plasma membrane occurs along microtubules, and nocodazole inhibits Fas translocation to plasma membrane by disrupting microtubules[12,13]. In the present study, nocodazole should have attenuated DR5 expression on the surface of detached cells, but DR5 expression remained unchanged in nocodazole-treated cells (Figure 1), indicating that microtubules are not involved in DR5 translocation.

The total DR5 expression level was 10-fold higher than the cell surface expression of DR5 in brefeldin-A-untreated EC9706 cells (Figure 2), demonstrating that the majority of total cellular DR5 was sequestered within intracellular compartments and the minority was associated with the plasma membrane.

DR5 localization in EC9706 cells

To further investigate the cellular localization and confirm the apparent translocation of DR5 from cell interior to plasma membrane, EC9706 cells were examined with immunostaining. In adherent cells, the results of both immunocytochemical and immunofluorescence staining showed that DR5 had a predominantly perinuclear

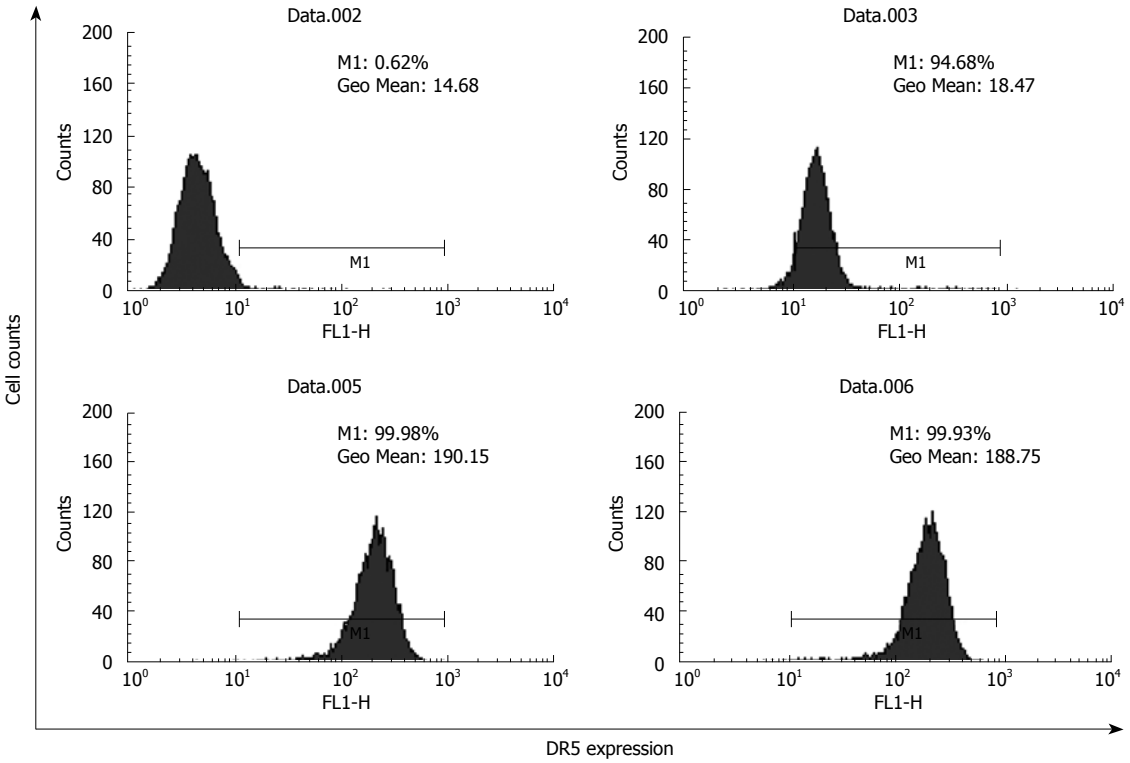

Figure 2 Analysis of total DR5 and extracellular expression in EC9706 cells by flow cytometry. Data.002: Isotype control; Data.003: Extracellular expression of DR5 in the cells pretreated without brefeldin A; Data.005: Total DR5 expression in the cells pretreated without brefeldin A; Data.006: Total DR5 expression in the cells pretreated with brefeldin A for 30 min before trypsinization.

localization but no nuclear localization (Figures 3A and 4A) and DR5 localization was not significantly different between brefeldin-A-treated and untreated cells (Figure 3A and B and Figure 4A and B). In detached cells, DR5 was predominantly localized in cytoplasm but not in nuclei (Figure 3C and D and Figure 4C and D). Furthermore, in brefeldin-A-untreated detached cells, DR5 tended to localize in the cell membrane and became exactly consistent with aggregation or capping of DR5 (Figure 4C). However, in brefeldin-A-treated cells, DR5 exhibited perinuclear localization (Figures 3D and 4D) and diffused (Figure 4D). These findings suggest that loss of cell adhesion enhanced DR5 translocation to the membrane of detached cells, leading to DR5 relocalization and aggregation.

Apoptosis in EC9706 cells

Since binding of DR5 agonist antibody mDRA-6 to DR5 triggers apoptotic cascades[10], we reasoned that by increasing cell surface expression of DR5, detached cells might become more susceptible to apoptosis induced by mDRA-6. Indeed, apoptosis induced by mDRA-6 was significantly increased in detached cells (Figure 5). The ability of detachment to sensitize EC9706 cells to apoptosis induced by mDRA-6 was inhibited by brefeldin A, suggesting that DR5 trafficking from cell interior to surface is necessary for detachment sensitization of EC9706 cells to mDRA-6-induced apoptosis. Interestingly, the spontaneous apoptotic rate for detached cells was higher than that for adherent cells ($P < 0.05$), demonstrating that increased cell surface expression of DR5 in detached cells is functional in transducing death signals and loss of cell adhesion may trigger apoptotic cascades *via* DR5 aggregation.

Activation of capsase-8 in EC9706 cells

It has been shown that TRAIL and anti-DR4/DR5 antibodies could trigger apoptotic signaling pathway of death receptor by activating caspase 8[2,10], which is implicated in apoptosis induced by cell detachment[7,14]. To characterize the signaling pathways through which detachment treatment substantially enhances mDRA-6-induced killing of EC9706 cells, activation of caspase 8 was determined by Western bloting in EC9706 cells. Consequently, time-dependent activation of caspase 8, as determined by decreased procaspase 8 (p55/53) levels and cleavage of caspase 8 (p43/p41), was observed after mDRA-6 treatment in spreading and temporarily detached EC9706 cells, respectively. Caspase 8 was activated 4 h earlier in temporarily detached cells than in spreading cells (Figure 6). However, even if the level of spontaneous apoptosis was much higher in temporarily detached EC9706 cells than in spreading EC9706 cells, caspase 8 activation was not detectable by Western bloting (data not shown), suggesting that activation of caspase 8 induced by cell detachment *via* DR5 oligomerization activation is very weak and is thus difficult to be assessed by Western bloting.

DISCUSSION

Since DR5 is the key death receptor of TRAIL[2-4], it is

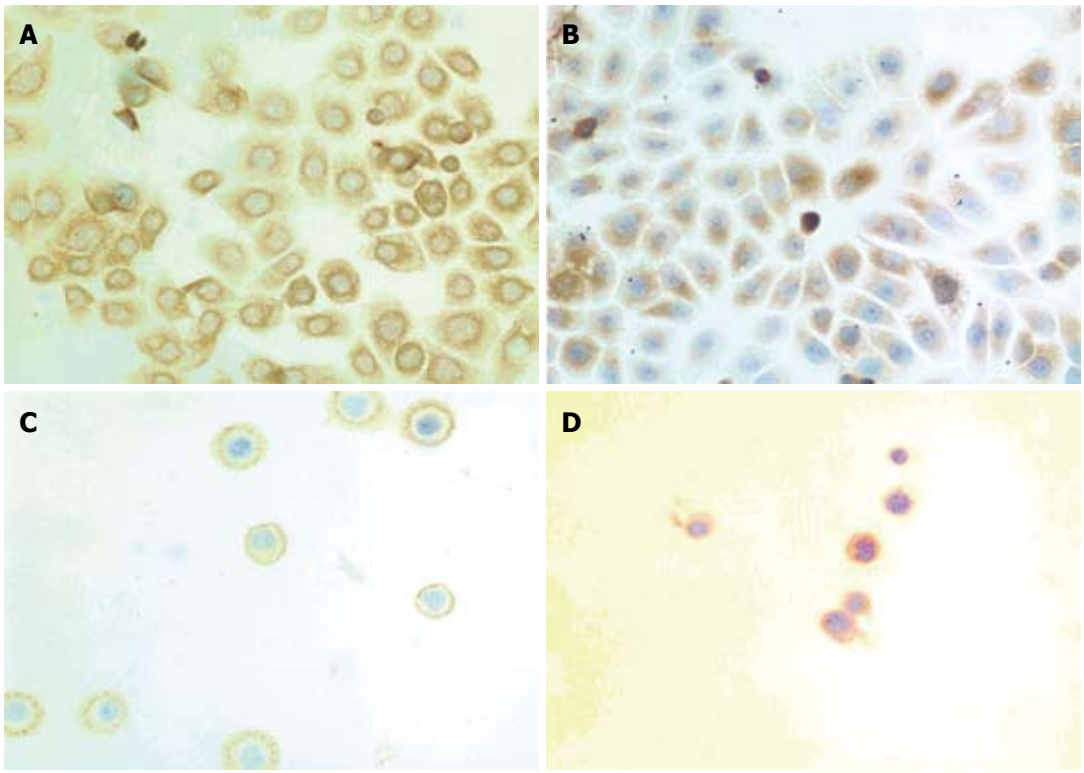

Figure 3 Immunostaining showing expression and localization of DR5 in EC9706 cells. A: Adherent cells pretreated without brefeldin A; B: Adherent cells pretreated with brefeldin A for 30 min; C: Detached cells not pretreated with brefeldin A before trypsinization; D: Detached cells pretreated with brefeldin A for 30 min before trypsinization (x 400).

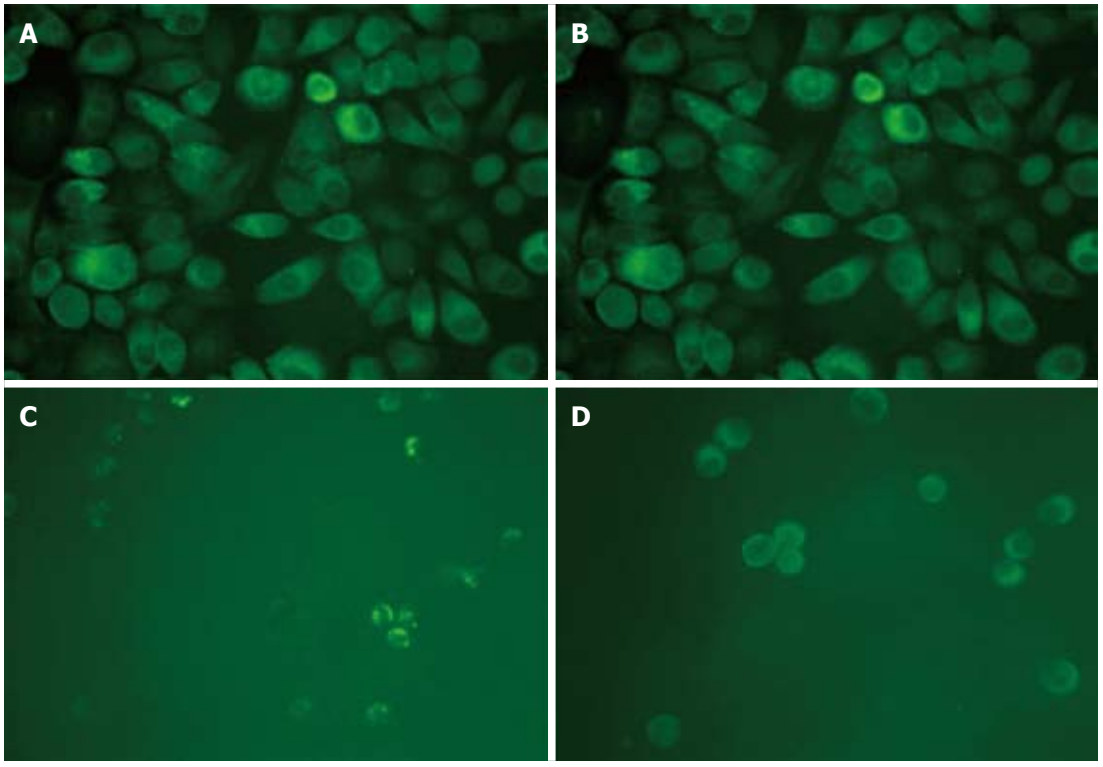

Figure 4 Immunofluorescence showing expression and distribution of DR5 in EC9706 cells. A: Adherent cells treated without brefeldin A; B: Adherent cells treated with brefeldin A before trypsinization; C: Detached cells not treated with brefeldin A; D: Detached cells treated with brefeldin A for 30 min before trypsinization (x 400).

very important to investigate its cellular expression and localization at the protein level. DR5, mainly localized in Golgi apparatus of cytoplasm, is a type of membrane protein[6] and translocated to cell membrane through the Golgi network. Brefeldin A can specifically inhibit the function of the Golgi apparatus[11]. This study showed

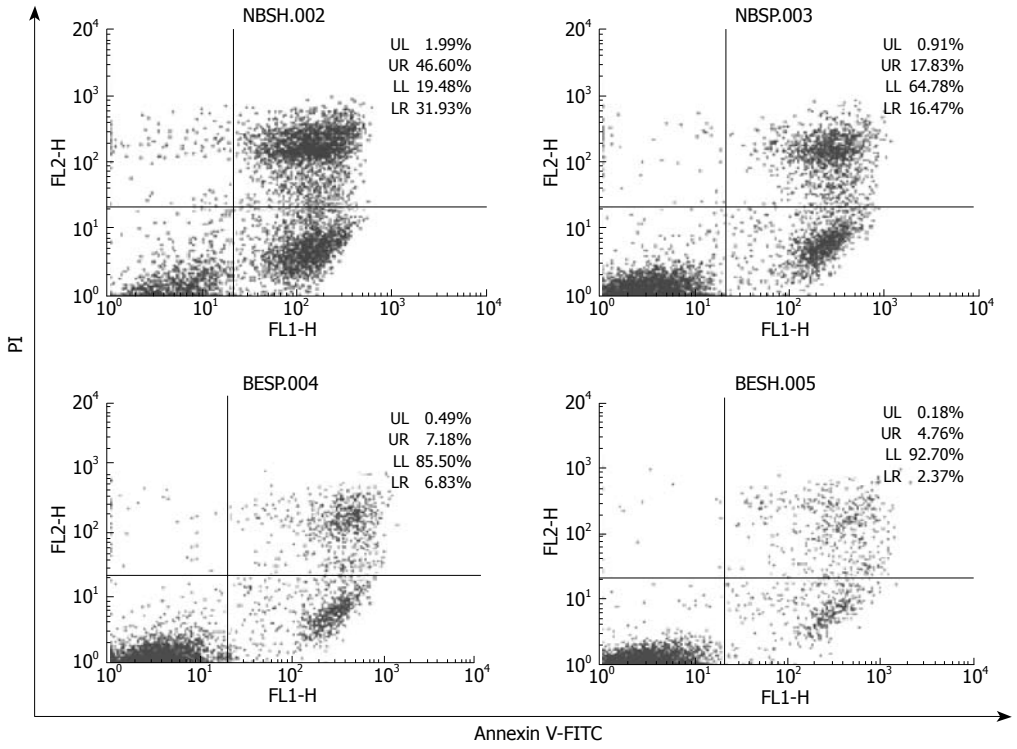

Figure 5 Flow cytomery showing apoptosis of EC9706 cells induced by anti-DR5 agonistic antibody. NBSH: Detached cells incubated with 2 mg/L mDRA-6 antibody for 12 h; NBSP: Spreading cells incubated with 2 mg/L mDRA-6 antibody for 12 h; BESH: Detached cells incubated in the medium without mDRA-6 antibody for 12 h; BESP: Spreading cells incubated in the medium without mDRA-6 antibody for 12 h.

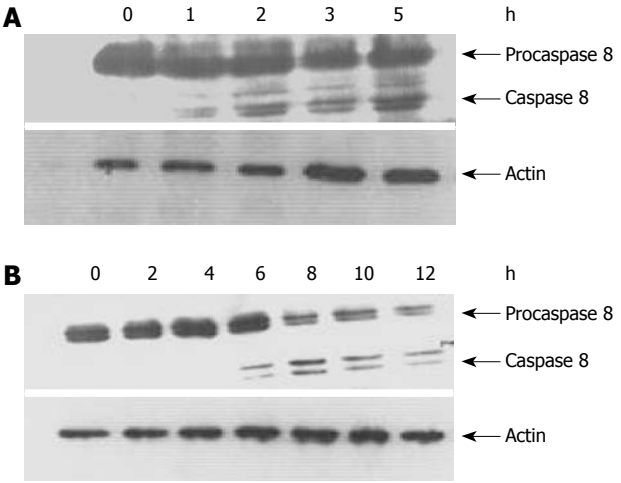

Figure 6 Activation of caspase 8 in EC9706 cells. A: After trypsinization, temporally detached EC9706 cells were incubated with agonist antibody mDRA-6 for an indicated time at 37℃; B: Spreading EC9706 cells were incubated with agonist antibody mDRA-6 for an indicated time at 37℃. Cell lysates were prepared and Western blotting was performed to analyze caspase 8 activation. The position of procaspase 8 and the active subunits were indicated. Western bloting for actin was utilized as the control of an equal sample loading.

that after EC9706 cells were treated with brefeldin A, the proportion of cells expressing DR5 on cell surface was significantly reduced in detached EC9706 cells, indicating that cell detachment is accompanied with translocation of DR5 from the cytoplasm to cell membrane (Figure 1), but a slight translocation occurred since DR5 was mainly localized in the cytoplasm (Figure 2), and total protein expression of DR5 did not increase in detached cells. However, it was recently reported that suspension culture enhances gene transcription and protein synthesis in DR5, but cell detachment does enhance DR5 expression on the cell surface[7]. Cell detachment from extracellular matrix had no effect on DR5 protein synthesis and the reason for this discrepancy was that cell detachment was temporary. To probe into DR5 translocation, morphological changes were observed with immunostaining in the present study. DR5 exhibited predominantly perinuclear localization in spreading EC9706 cells, while DR5 receptors were localized near the cell membrane of detached cells (Figures 3 and 4), revealing that cell detachment is mainly responsible for intracellular relocalization of DR5 and promotes translocation of DR5 to the cell membrane. It has been shown that DR5 receptors are mainly localized in the plasma membrane of some suspension cells[15], and in the cytoplasm of some spreading cells[6,16], suggesting that cellular localization of DR5 is closely related to the growth pattern and adhesion status of cells.

It was reported that the Golgi complex and cytoskeletal system are directly involved in translocation of membrane proteins and secretory proteins[11,13,17]. Cell detachment influences cellular localization of DR5 and promotes translocation of DR5 to the cell membrane, indicating that the cytoskeleton may be involved in cellular localization and translocation of DR5. It has been shown that glycyl-chenodeoxycholic acid may enhance Fas expression on the surface of human liver cells, but nocodazole which can disrupt microtubules, inhibits the function of bile acids, suggesting that microtubules are

involved in translocation of Fas[13]. However, results of the current study show that there was no difference in DR5 expression rate and intensity between nocodazole-treated and untreated esophageal cancer cells (Figure 1), demonstrating that microtubules are not implicated in DR5 translocation. The reason for this discrepancy still remains unclear and the inconsistent results might be attributed to the types of death receptors, cells, and stimulating period of time.

It has been shown that DR5 expression on the surface of tumor cells is closely related to the sensitivity of tumor cells to TRAIL-induced apoptosis[2,3]. Therefore, increased DR5 expression on the cell surface might be accompanied with increased cell sensitivity to TRAIL-induced apoptosis. mDRA-6 is an anti-DR5 agonist antibody and exhibits tumoricidal activities by inducing apoptosis[10]. This study demonstrated that when spreading esophageal cancer cells were transformed to detached cells, the rate of mDRA-6-induced apoptosis was increased by 44.23% (Figure 5). Although this was definitely attributed to the high cell-surface-expression levels of DR5 caused by cell detachment, it was more likely due to the aggregation of DR5. Loss of adhesion in esophageal cancer cells was found to be responsible for the granular distribution of DR5 in these cells (Figures 3 and 4), indicating that cell detachment leads to DR5 aggregation. Moreover, it was reported that DR5 aggregation might cause ligand-independent activation of DR5, thus resulting in activation of caspase 8 and consequently apoptosis[18]. It was also reported that detachment could activate caspase-8, leading to anoikis[14]. It has been recently shown that activation of caspase-8 mediated by detachment contributes prominently to DR5[7]. In this study, the spontaneous apoptotic rate for detached EC9706 cells was higher than that for adherent cells ($P < 0.05$) (Figure 5), and caspase 8 was activated 4 h earlier in temporarily detached cells than in spreading detached cells in the presence of DR5 agonist antibody (Figure 6), suggesting that cell detachment may lead to activation of caspase 8 due to DR5 aggregation. However, Western blot analysis revealed that no activated caspase 8 was detected in temporally detached EC9706 cells in the absence of DR5 agonist antibody (Figure 6), and it seems that the period of cell detachment was not long enough, so that activation of caspase 8 induced by DR5 oligomerization was too weak to be detected by Western bloting. DR5 oligomerization activation may exist in detached cells, thus effectively reducing the threshold of apoptosis, which increases the apoptotic sensitivity of detached cells to DR5 agonist antibody.

Cellular localization of DR5 may influence cell survival, and may change from cell adhesion to cell detachment. Since most types of human cells exhibit adhesion growth, DR5 receptors are mainly located in the cytoplasm, and only a few DR5s are present on the cell surface. Cytoplasmic localization of DR5 effectively reduces its interaction with TRAIL, which may lead to apoptosis, thus leading to cell survival. Furthermore, high levels of TRAIL have been reported in tumor-infiltrating lymphocytes of cancer patients[19], and DR5 maybe highly express on the surface of detached tumor cells. Thus, interaction between tumor and immune cells may increase apoptosis of metastatic cells, and inhibit metastasis of tumor cells. Consequently, low expression of DR5 augments tumor metastasis. In addition, if DR5 receptors are mainly located in the cytoplasm of adherent cells, the localization of DR5 may lead to resistance of tumor cells to TRAIL-induced apoptosis and prevent tumor cells from TRAIL-induced apoptosis. However, some drugs can up-regulate DR5 expression and enhance translocation of DR5 to the cell membrane[18,20], and these drugs may increase the sensitivity of tumor cells to TRAIL-induced apoptosis and enhance the efficacy of TRAIL-induced apoptosis. Thus, TRAIL or agonistic anti-DR5 antibody in combination with subtoxic doses of chemotherapeutic agents and radiotherapy may provide a promising effective therapeutic strategy for resistant tumors.

In summary, detachment of EC9706 cells from extracellular matrix causes DR5 relocalization, promotes cytoplasmic translocation of DR5 to the cell surface *via* a Golgi-dependent pathway, and also results in DR5 oligomerization and apoptosis. Our findings provide new insights into the mechanisms underlying the regulation of intracellular localization and translocation of DR5, and also show the potential clinical applications of DR5 antibodies and TRAIL in anticancer therapy. How cellular detachment influences DR5 translocation and aggregation needs to be further studied. Furthermore, additional works are needed to determine whether DR5 expression varies with the adherent phase of cells in all solid tumors.

COMMENTS

Background

DR5, the main agonist death receptor of TRAIL, is a good potential target for anti-tumor therapy. Since TRAIL and agonistic antibody against death receptor 5 (DR5) can activate membrane-bound DR5 to trigger apoptosis, cellular localization of DR5 receptors directly affects TRAIL-induced apoptosis. In the development and progress of malignant tumors, detachment from primary tumor is an obligatory step in metastasis; tumor cells must detach from their substrates at a distant site to create metastasis. To date, DR5 expression caused by cell detachment remains poorly understood. In the present study, the effect of cell detachment on DR5 relocalization and translocation in esophageal carcinoma cells was observed.

Research frontiers

This study was focused on the relocalization and translocation of DR5 and apoptosis during the process of cell detachment.

Applications

This study showed that detachment of EC9706 cells from extracellular matrix caused DR5 relocalization, promoted cytoplasmic translocation of DR5 to the cell surface *via* a Golgi-dependent pathway, and also resulted in DR5 oligomerization. These findings will provide new insights into the mechanisms underlying DR5 translocation regulation and anoikis, and also indicate the potential clinical applications of DR5 antibodies and TRAIL in anticancer therapy.

Peer review

The authors investigated the potential effect of detachment of esophageal squamous carcinoma cells from extracellular matrix on the localization and translocation of DR5, and the results demonstrate that detachment of EC9706 cells from extracellular matrix could cause DR5 relocalization, promote cytoplasmic translocation of DR5 to the cell surface *via* a Golgi-dependent pathway, and result in DR5 oligomerization. The study was well designed with interesting and informative findings.

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BRIEF ARTICLES

Correlation analysis of celiac sprue tissue transglutaminase and deamidated gliadin IgG/IgA

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Author contributions: Marietta EV, Rashtak S and Murray JA designed research; Rashtak S performed research; Marietta EV, Rashtak S and Murray JA analyzed data; Marietta EV wrote the paper.
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Abstract

AIM: To indirectly determine if tissue transglutaminase (tTG)-specific T cells play a crucial role in the propagation of celiac disease.

METHODS: Anti-deamidated gliadin peptide (DGP) and anti-tTG IgA and IgG were measured in the sera of celiac patients (both untreated and treated). The correlations were determined by Spearman's rank correlation test.

RESULTS: In celiac patients, we found a very significant correlation between the production of DGP IgA and IgG (r = 0.75), indicating a simultaneous and ongoing production of these two isotypes reminiscent of oral vaccination studies. However, there was far less association between the production of tTG IgA and tTG IgG in celiac patients (r = 0.52). While tTG IgA was significantly correlated with DGP IgA (r = 0.80) and DGP IgG (r = 0.67), there was a weak correlation between production of anti-tTG IgG and the production of anti-DGP IgA (r = 0.38) and anti-DGP IgG (r = 0.43).

CONCLUSION: These data demonstrate that the production of anti-tTG IgA is directly correlated to the production of anti-DGP IgG and IgA, whereas anti-tTG IgG is only weakly correlated. This result therefore supports the hapten-carrier theory that in well-established celiac patients anti-tTG IgA is produced by a set of B cells that are reacting against the complex of tTG-DGP in the absence of a tTG-specific T cell.

Key words: Celiac disease; Tissue transglutaminase; Deamidated gliadin peptide; Correlation; IgG; IgA

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INTRODUCTION

Celiac disease is a gluten-sensitive disease that afflicts primarily the small bowel, resulting in the shortening of villi, increased numbers of intraepithelial lymphocytes, and crypt hyperplasia[1,2]. One unique feature of celiac disease that is utilized as a diagnostic and screening tool is the production of IgA specific for tissue transglutaminase (tTG) that circulates in the blood[3]. It is unclear though, as to why these antibodies are generated when a celiac patient eats gluten. One association between tTG and gliadin is that intestinal T cells from celiac patients respond to specific gliadin peptides that have been deamidated, a process that is mediated by tTG binding to gliadin peptides[4]. Anti-tTG IgA is tightly associated with the development of enteropathy and brings into question whether it is a cause or consequence of enteropathy[5,6]. It is especially perplexing because many celiac patients will produce IgA against whole gliadin, a storage protein of gluten, yet this production has a much lower specificity for celiac disease than the anti-tTG IgA ELISA assay[7].

One theory for the origin of anti-tTG IgA was proposed in 1997[8]. This proposal was based on a hapten-carrier model wherein gliadin-specific T cells contribute to the stimulation of B cells that are specific for tTG. This would be achieved by the tTG-specific B cells internalizing complexes of tTG and gliadin peptides and later

presenting gliadin epitopes to the gliadin-specific T cells. In this manner, gliadin-specific T cells would contribute to the tTG-specific B cells producing antibodies against tTG. At that time, this proposal was supported by a lack of evidence for tTG-specific T cells. It is notable that to this date, there is still no evidence that a tTG-specific T cell exists in the small intestine of celiac patients. Of course, it is difficult to prove the absence of a cell type. However, novel ELISAs have been recently developed that can detect antibodies against deamidated gliadin peptides (DGP) in celiac patients[9,10]. This allows us to look at this process in an indirect manner, by determining the correlation among the production of antibody isotypes against DGP and tTG.

MATERIALS AND METHODS

Subjects and study design

Serum samples were collected from patients referred to the division of Gastroenterology and Hepatology at the Mayo Clinic, Rochester, MN, USA for the assessment of gastrointestinal symptoms, unexplained weight loss/anemia, or to rule out celiac disease. One hundred and twenty-one celiac patients were initially included in the study. We defined the diagnosis of celiac disease based on the presence of villous atrophy (enteropathy type ⅢA or greater based on currently accepted diagnostic criteria) in histopathological examination of small intestinal biopsy[11,12]. Of 121 celiac patients who were initially included in the study, 10 were excluded because they had Marsh I enteropathy (n = 8) or IgA deficiency (n = 2). One hundred and ninety-four serum samples were collected from the remaining 111 biopsy-proven celiac patients. Ninety-two samples were collected before patients started treatment and 102 samples were collected while patients were on a gluten-free diet (GFD). The median (range) treatment with GFD was 10.5 (2-54) mo. The study was approved by the Institutional Review Board of Mayo Clinic.

Serology

Anti-DGP IgG and IgA were measured with "QUANTA Lite Gliadin-IgA Ⅱ and Gliadin-IgG Ⅱ" ELISA kits (INOVA Diagnostics Inc., San Diego, CA, USA). Anti-tTG IgA and IgG were measured using "BINDAZYME human IgA and IgG Anti-Tissue Transglutaminase EIA" ELISA kits (The Binding Site, Ltd., Birmingham, UK).

Statistical analysis

Correlations between the antibody titers were assessed by Spearman's rank correlation coefficients that were calculated using version 6.0.0 JMP software (SAS Institute Inc., Cary, NC, USA).

RESULTS

The production of IgA and IgG specific for DGP and tTG was evaluated in celiac patients and plotted such that a direct comparison was made between the production of IgG versus IgA for each antigen group and each patient group (Figure 1). There was a significantly stronger correlation between the production of IgA and IgG specific for DGP (r = 0.75) in celiac patients than those specific for tTG (r = 0.52). When untreated celiac patients (gluten-containing diet; GCD) were separated from treated celiac patients (GFD), the correlation coefficients in comparing anti-DGP IgG and IgA were 0.78 for GCD and 0.58 for GFD, whereas a significantly lower correlation was found for comparing anti-tTG IgG and IgA (r = 0.60 for GCD and r = 0.44 for GFD).

Comparisons were also made between the production of anti-tTG IgA and the production of DGP IgA and IgG in celiac patients (Figure 2). Anti-tTG IgA was highly correlated with the production of both anti-DGP IgA (r = 0.80) and DGP IgG (r = 0.67) which was similar to a previous finding[9].

Finally, comparisons were made between the production of anti-tTG IgG and the production of IgA and IgG specific for DGP. In contrast to anti-tTG IgA which was strongly correlated with DGP antibodies, anti-tTG IgG was weakly correlated with the production of anti-DGP IgA (r = 0.38) and anti-DGP IgG (r = 0.43).

DISCUSSION

The data presented in this manuscript support the theory that the generation of anti-tTG IgA is directly linked to the B cell immune response against DGP, possibly even the T-cell immune response to DGP as well. The reduced correlation in celiac patients between the production of anti-tTG IgG and anti-tTG IgA (r = 0.52) as compared to the production of anti-DGP IgG and anti-DGP IgA (r = 0.75) also demonstrates that there is a fundamental difference between the generation of antibody isotypes against the two antigens in celiac patients. Another difference between the production of IgG and IgA against DGP and tTG is that dietary gliadin mainly affects the production of both IgG and IgA against DGP, but not against both tTG IgG and IgA.

The lack of correlation between the production of anti-tTG IgG and anti-DGP IgG and IgA and anti-tTG IgA (Figures 1 and 2) therefore raises several questions. If the inflammatory T cells that are specific for deamidated gliadin are providing help to the B cells that are specific for a tTG/gliadin complex, why are not more tTG-specific B cells isotype class switching to IgG? One explanation is that the tTG/gliadin-specific B cell group in well-established celiac patients is fully committed to being IgA-positive. A memory B cell could be one such type of long lived IgA-positive B cell, and in this way, require minimal help from a bystander T cell response in order to be fully activated. It is notable that treated celiac patients will relapse with a gluten challenge, even after years of adhering to a GFD, indicating that there is a strong memory component of T cells, B cells, or both in celiac patients[13-15].

Also of interest are studies that demonstrated significant variability in the production of anti-tTG

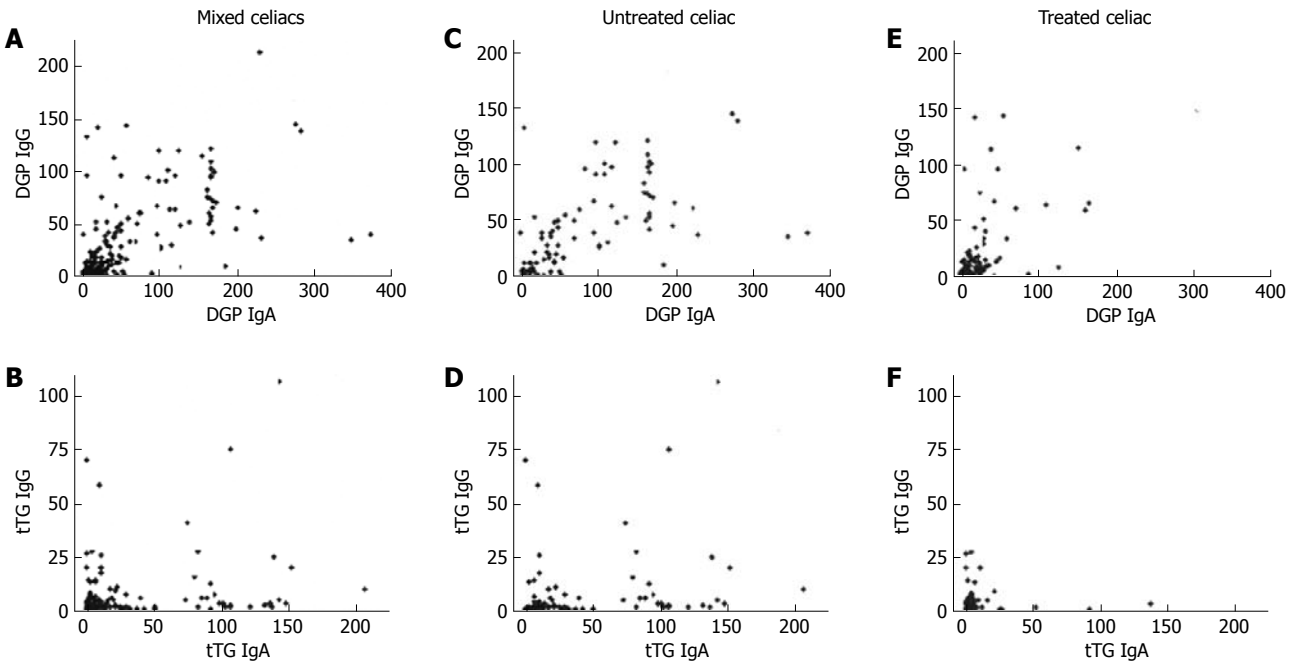

Figure 1 Effect of diet upon isotype correlations. The titers of IgG and IgA against DGP and tTG were evaluated and plotted against each other for celiac patients. For mixed (treated and untreated) celiac patients, the Spearman's rank correlation coefficients were $r = 0.75$ for DGP (A) and $r = 0.52$ for tTG (B). For untreated celiac patients, $r = 0.78$ for DGP (C) and $r = 0.60$ for tTG (D). For treated celiac patients, $r = 0.58$ for DGP (E) and $r = 0.44$ for tTG (F).

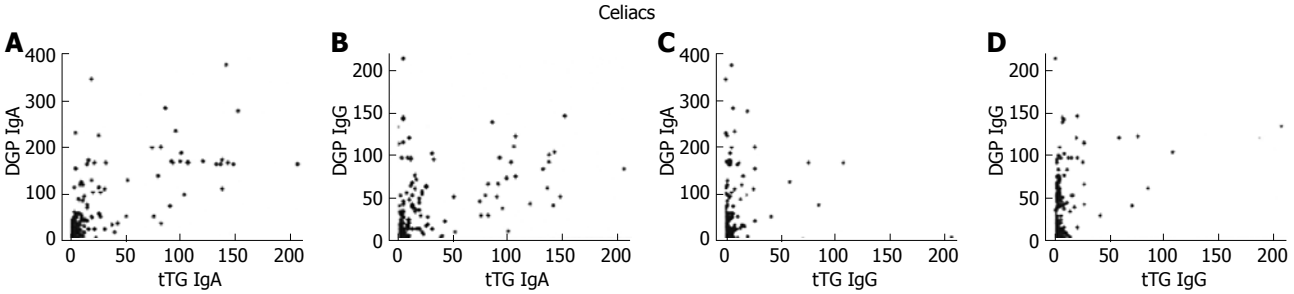

Figure 2 Comparing anti-tTG IgA and IgG production with anti-DGP IgA and IgG. The titers of anti-tTG IgA (A-B) and anti-tTG IgG (C-D) were compared with the titers of anti-DGP IgA (A and C) as well as anti-DGP IgG (B and D) in all treated and untreated celiac patients. Spearman's rank correlation coefficients were 0.80 (A), 0.67 (B), 0.38 (C), and 0.43 (D).

IgA in children. One study found that only six out of 14 celiac patients less than 2 years of age had anti-endomysial antibodies[16]. Another group reported markedly fluctuating levels of transglutaminase antibodies in children[17]. These data would indicate that anti-tTG antibodies do not develop immediately at the time of initial exposure to dietary gliadin, but instead develop after 1-2 (or more) years of continued gliadin exposure.

Our data, as well as the data from others, therefore support the hapten-carrier theory that a B cell that is specific for a tTG/gliadin complex is present in well-established celiac patients and is helped by gliadin-specific T cells. However, our data are also compatible with the model based on molecular mimicry between tTG and gliadin[9,18]. Indeed, it is our belief that the hapten-carrier model and molecular mimicry model are not exclusive. A potential "combined" model would be that a catalyst-like IgA+ memory B cell specific for regions that are shared between tTG and gliadin exists long term in well-established celiac patients. With the consumption of gliadin, these B cells would internalize tTG/gliadin complexes, become activated with minimal T cell help, and then present gliadin peptides to gliadin-specific T cells. This would result in the amplification of both deamidated gliadin specific T- and B-cell responses, as well as the production of anti-tTG IgA antibodies.

COMMENTS

Background

The origin of anti-tissue transglutaminase (tTG) IgA in celiac disease has proven to be elusive and currently two theories exist. One theory is a hapten-carrier model, whereby gliadin-specific T cells provide help for tTG-specific B cells. The other is based on molecular mimicry between tTG and gliadin.

Research frontiers

The recent detection of antibodies in celiac patients specific for deamidated gliadin peptides (DGP), the product of tTG binding to gliadin peptides, provides an opportunity to address the correlation between the production of anti-tTG IgA and the antibodies against DGP in celiac patients.

Innovations and breakthroughs

This study has made the novel observation that the production of both IgG and

IgA against DGP is significantly correlated with the production of anti-tTG IgA and weakly with anti-tTG IgG. This would indicate that the T and B cell response against DGP is fundamentally different from the T- and B-cell response against tTG, and would therefore support the hapten-carrier theory of the origin of tTG IgA.

Applications

By determining the origin of anti-tTG IgA in celiac disease, we obtain a better understanding of the (potentially pathogenic) role of anti-tTG IgA in the development of celiac disease.

Terminology

DGP and tTG are terms that refer to deamidated gliadin peptides and tissue transglutaminase, respectively. Also, gluten free diet (GFD) and GCD refer to gluten-free diet and gluten-containing diet.

Peer review

The data presented in this rapid communication are of interest to the celiac disease community. It is a rapid communication that examines the pattern of serum IgG and IgA levels specific to DGP and tTG in celiac disease patients. It also determines how the administration of a GFD therapy affects this pattern.

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Epidemiology of hepatitis B virus infection in Albania

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Abstract

AIM: To assess the prevalence and socio-demographic distribution of hepatitis B virus (HBV) infection in Albania.

METHODS: Blood samples from 410 unselected schoolboys, 666 students, 500 military personnel, 1286 casual blood donors, 378 voluntary blood donors and 640 pregnant women (total 3880 non-vaccinated residents of rural and metropolitan areas from all over Albania; 2354 (60.7%) male and 1526 (39.3%) female; mean age of 26.3 years) were tested during 2004-2006 for hepatitis B surface antigen (HBsAg) and antibodies to hepatitis B virus (anti-HBs) by ELISA.

RESULTS: The HBsAg and anti-HBs prevalence were 9.5% and 28.7%, respectively. The highest HBsAg prevalence was evident in the younger age group, such as in schoolchildren (11.8%) and the military (10.6%). Consequently, the anti-HBs prevalence increased with age, from 21.2% in schoolchildren (mean age: 15.7 years), to 36.3% in pregnant women (mean age: 26.3 years) and 29.7% in voluntary blood donors (mean age: 40.1 years). There were no significant differences between males and females.

CONCLUSION: Despite the estimated two-fold reduction of HBsAg prevalence in the general population from about 18%-19% to 9.5%, Albania remains a highly endemic country (i.e. over 8% of HBsAg prevalence rate).

Key words: Albania; Hepatitis B virus; Blood donor; Military; Pregnant women; Schoolchildren; Student

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INTRODUCTION

Hepatitis B is a disease of global distribution. It is estimated that about 30% of the world's population, i.e. approximately 2 billion people, show serological evidence of hepatitis B virus (HBV) infection and about 40 million are persistent carriers of HBV [1]. Each year over one million people die from HBV-related chronic liver disease, including cirrhosis and hepatocellular carcinoma[2].

The endemicity of HBV infection varies greatly worldwide and is influenced primarily by the age at which infection occurs[3,4]. In Europe, the level of endemicity increases from north to south and from west to east. Most countries of northern and western Europe have a very low prevalence of HBV infection (less than 0.5% of the population being positive for HBsAg). Unexpectedly high prevalence of hepatitis B carriage (5%-12%) have been found in many parts of central and Eastern Europe and the former Soviet Union countries[5,6]. Endemicity of infection is considered high in those parts of the world where at least 8% of the population is HBsAg positive. Almost all infections

occur either during the prenatal period or early in a childhood, which accounts for the high rates of chronic HBV infection in these populations[7].

Credible epidemiological data of HBV infection in Albania, before the introduction of obligatory vaccination of newborn children against HBV (1995), was obtained by screening Albanian refugees during the first mass scale migration from Albania to Italy and Greece that occurred in 1991[8-10]. Although the refugees represented mostly subjects from lower socio-economic classes, the large number of people enrolled from different geographic areas (rural and urban) provided important information on HBV infection in Albania (Table 1). The presence of one or more serological markers of HBV infection and the high rate of infection in children aged 1 to 10 years confirms the endemic nature of this virus in Albania.

The above-mentioned data of HBV infection in Albania were undoubtedly related to low hygiene and poor economic situation, overcrowded conditions, lack of disposable needles and syringes, lack of safe blood and its products for transfusion, inadequate sterilization of reusable equipment, difficulties in obtaining appropriate personal equipment to prevent exposure to blood, and lack of an immunization program against HBV.

In 1992, WHO recommended that all countries should include hepatitis B vaccine in their routine infant immunization programs. Since May 1995, thanks to the Rotary International Club, Albania introduced vaccination of newborn children against HBV into the National Immunization Programs as the most appropriate immunization strategy to reduce the rate of HBV infection and HBV-related chronic liver diseases. Infants are immunized at birth, and then after 1 and 5 mo.

MATERIALS AND METHODS

Blood samples from 3880 randomly selected non-vaccinated residents of rural and urban areas from all over Albania were tested during 2004-2006 for HBsAg and anti-HBs by ELISA The blood samples were obtained from 2354 (60.7%) males and 1526 (39.3%) females (mean age of 26.3 years) comprising 410 schoolchildren, 666 students, 500 military, 1286 casual blood donors, 378 voluntary blood donors and 640 pregnant women. Casual blood donors included individuals who donated blood only once, whereas voluntary blood donors included regular blood donors (Table 2). We took blood samples randomly from schoolchildren from several high schools, students from the University of Tirana and soldiers from several military units in main districts of Albania. We also collected blood samples from all casual blood donors and voluntary blood donors during 2004-2005 at the Blood Bank Centre of Tirana. The origin of the subjects was approximately equally distributed between rural and urban regions (1834 rural, 1846 urban).

RESULTS

Baseline characteristics of the study population are presented in Table 2.

Table 1 Prevalence of hepatitis B markers in Albanian refugees according to studies in Italy and Greece

Author	Sanantonio *et al*	Dalekos *et al*	Malamitsi-Puchner *et al*
Study region	Bari	Ioannina	Athens
Yr	1993	1995	1996
Ages	Adults	All ages	Pregnant women
No. cases	393	1025	500
% prevalence of HBsAg	19	22.2	13.4
% prevalence of anti-HBs	55	52	53

Table 2 Baseline characteristics of the study groups

Study groups	Characteristics			
	No (%)	M/F (%)	Mean age (yr)	Yr
Schoolchildren	410 (10.6)	264 (64.4)/ 146 (35.6)	15.7 ± 1.2	2004
Students	666 (17.2)	340 (51.1)/ 326 (48.9)	23.1 ± 1.7	2005
Military	500 (12.9)	500 (100)/ 0 (0)	19.2 ± 2.3	2005
Casual blood donors	1286 (33.1)	987 (76.6)/ 299 (23.3)	32.4 ± 4.8	2004
Voluntary blood donors	378 (9.7)	263 (69.6) 115 (30.3)	40.1 ± 5.1	2005
Pregnant women	640 (16.5)	0 (0) 640 (100)	27.4 ± 4.9	2006
Total	3880	2354 (60.7) 1526 (39.3)	26.3 ± 6.2	2004-2006

Table 3 HBsAg and anti-HBs prevalence in different study groups

Study groups	No. cases	Prevalence (%)	
		HBsAg-positive	antiHBs-positive
Schoolchildren	410	48 (11.8)	87 (21.2)
Students	666	58 (8.7)	247 (37.2)
Military	500	54 (10.6)	124 (24.7)
Casual blood donors	1286	115 (8.9)	293 (22.8)
Voluntary blood donors	378	36 (9.6)	112 (29.7)
Pregnant women	640	47 (7.3)	232 (36.3)
Total	3880	358 (9.5)	1095 (28.7)

The HBsAg and anti-HBs prevalence was 9.5% and 28.7%, respectively. The highest HBsAg prevalence rate was evident in the younger age groups, such as in schoolchildren (11.8%) and in military personnel (10.6%). Consequently, the anti-HBs prevalence increased with age, from 21.2% in schoolchildren (mean age: 15.7 years), to 37.2% in students (mean age: 23.1 years), to 36.3% in pregnant women (mean age: 26.3 years) and 29.7% in voluntary blood donors (mean age: 40.1 years). There were no significant differences between males and females (Table 3). With egard to the age groups, we found prevalence of HBsAg was: 16-20 years: 11.8%; 21-25 years: 9.2%; 26-30 years: 8.3%; 31-35 years: 8.9%; 36-40 years: 9.5%; 41-45 years: 9.5%. We found higher prevalence of HBsAg positivity in urban inhabitants compared with rural inhabitants (11.8% and 7.6%, respectively).

DISCUSSION

The data of this study showed an evident reduction of HBsAg in the general non-vaccinated population of Albania, from 18%-19% (before 1995) to 9.5%. Similar HBsAg prevalence rates were noted among pregnant Albanian women delivering in Greece, and in Albanian health care workers (9.8% and 8.1%, respectively)[11,12].

The success of routine immunization of children and adolescents in interrupting HBV transmission has been previously demonstrated in several high- and low-endemic areas[7]. A primary indicator of the positive impact of hepatitis B vaccination is a reduction of the seroprevalence of HBsAg in the vaccinated population[13]. HBsAg carrier rate in the vaccinated groups has decreased by as much as 74% in less than 10 years in Italy, 96% in 7 years in Saudi Arabia, 93% in 15 years in Taiwan, 79% in 10 years in Thailand, and almost 100% in Alaska[14-18]. Apart from the decreasing seroprevalence of HBsAg in vaccinated populations, another indicator is the decline in the number of acute cases of hepatitis B. Although infections in pediatric age groups are not easy to demonstrate because hepatitis B is rarely symptomatic, trends in the incidence of acute hepatitis B disease can be used to evaluate the influence of vaccination programs in adolescents and adults who are most likely to have asymptomatic infections after HBV exposure[19,13]. In countries such as Italy and the United States, the incidence of acute hepatitis B has declined dramatically during the last decade, particularly among young age groups[20,21]. A significant decline of annual frequency of acute viral hepatitis B from 692 new cases in 2000, to 348 in 2005 was also noted in Albania[22].

Taking into consideration: (1) the reinforcement of the general preventive measures, such as the implementation of the safe injection procedures, proper sterilization of the medical and dental equipment, proper screening of the blood and its products, and progress in health education; and (2) vaccination of some high-risk groups (health care workers, hemodialysis and thalassemic patients), the significant reduction of HBV markers among the non-vaccinated general population (9.5%) compared to the previous rate of 1993-1995 (18%-19%), may be attributed to the 12 consecutive years of vaccination of newborn children against HBV. Similar decreases in HBsAg carrier rates in the non-vaccinated population were also observed in Saudi Arabia and Taiwan[23,24].

The main cause of the reduction in HBsAg prevalence in the general non-vaccinated population (after infant vaccination against HBV) is based on the effective prevention of perinatally transmitted HBV infections among children of HBsAg-positive mothers, and prevention of early childhood transmission between household contacts, which are thought to be responsible for a significant number of HBV infections[18,25-29]. Even in regions with low endemicity, transmission of infection between children and transmission from infected infants to adults has been well documented. This risk of transmission is also demonstrated by the higher infection rate in refugee families and in children's institutions[26]. Furthermore, chronically infected children are likely to be HBeAg positive with a high infectious potential for transmission to other children or adults. Thus, we hypothesize that vaccination programs decrease the risk of HBV infection not only for vaccinated children, but also for all of the population, even those who are non-vaccinated.

COMMENTS

Background

Hepatitis B is a disease of a global distribution. The epidemiological situation of hepatitis B virus (HBV) infection in Albania before the introduction of obligatory vaccination on newborn children against HBV in 1995 was very grave, with high prevalence rates of HBsAg in general population.

Research frontiers

Despite the estimable two-fold reduction of HBsAg prevalence in general population from about 18%-19% to 9.5%, Albania remains a high endemic country.

Innovations and breakthroughs

The vaccination program of newborn children against HBV infection has beneficial effects in the decrease of HBsAg prevalence in non vaccinated population.

Peer review

It is in general well written, organized and interesting.

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Serum biomarker tests are useful in delineating between patients with gastric atrophy and normal, healthy stomach

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Abstract

AIM: To study the value of serum biomarker tests to differentiate between patients with healthy or diseased stomach mucosa: i.e. those with *Helicobacter pylori (H pylori)* gastritis or atrophic gastritis, who have a high risk of gastric cancer or peptic ulcer diseases.

METHODS: Among 162 Japanese outpatients, pepsinogen Ⅰ (Pg Ⅰ) and Ⅱ (Pg Ⅱ) were measured using a conventional Japanese technique, and the European GastroPanel examination (Pg Ⅰ and Pg Ⅱ, gastrin-17 and *H pylori* antibodies). Gastroscopy with gastric biopsies was performed to classify the patients into those with healthy stomach mucosa, *H pylori* non-atrophic gastritis or atrophic gastritis.

RESULTS: Pg Ⅰ and Pg Ⅱ assays with the GastroPanel and the Japanese method showed a highly significant correlation. For methodological reasons, however, serum Pg Ⅰ, but not Pg Ⅱ, was twice as high with the GastroPanel test as with the Japanese test. The biomarker assays revealed that 5% of subjects had advanced atrophic corpus gastritis which was also verified by endoscopic biopsies. GastroPanel examination revealed an additional seven patients who had either advanced atrophic gastritis limited to the antrum or antrum-predominant *H pylori* gastritis. When compared to the endoscopic biopsy findings, the GastroPanel examination classified the patients into groups with "healthy" or "diseased" stomach mucosa with 94% accuracy, 95% sensitivity and 93% specificity.

CONCLUSION: Serum biomarker tests can be used to differentiate between subjects with healthy and diseased gastric mucosa with high accuracy.

Key words: Gastric atrophy; *Helicobacter pylori*; Serum gastrin-17; Serum pepsinogen

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INTRODUCTION

In 1994, the International Agency on Research for Cancer (IARC) considered *Helicobacter pylori (H pylori)* infection to be a class I carcinogen[1]. *H pylori* infection results in chronic gastritis that will develop into atrophic gastritis of some grade or type in half of infected subjects during their lifetime[2,3]. *H pylori* itself is not carcinogenic but the gastritis it causes, particularly atrophic gastritis, and the subsequent hypochlorhydric

stomach are carcinogenic[1,4-9]. On the other hand, subjects with normal, healthy stomach mucosa have no significant cancer risk, and are also not at risk for peptic ulcer diseases except those who use aspirin or NSAIDs[10]. Therefore, the differentiation between patients with healthy (no *H pylori*, gastritis or atrophic gastritis) and diseased gastric mucosa is clinically relevant. From the viewpoint of cost-effectiveness, this differentiation may be helpful in clinical decision-making and in rationalizing and optimizing diagnostic, therapeutic and screening procedures[11-15].

In the diagnosis of atrophic gastritis, and in the differentiation between healthy and diseased stomach mucosa, two options are available. The first option is gastroscopy and microscopic examination of endoscopic biopsy from the gastric antrum and corpus. The second non-invasive option, is the examination of gastric biomarkers from serum or plasma. Serum levels of pepsinogen (Pg) have been used for decades to diagnose atrophic corpus gastritis non-invasively[16-21]. In particular, in Japan, a country known to have a high prevalence of *H pylori* infection accompanied by gastric atrophy, the usefulness of the serum test to diagnose gastric atrophy has been extensively investigated[22-26], and there has been some success in screening subjects with a high risk of gastric cancer by determining the serum Pg I and Pg I / II ratio[8,27]. Recently, a European biomarker examination, GastroPanel (Biohit Plc, Helsinki, Finland), which not only assays Pg levels but also measures serum or plasma levels of gastrin-17 (G-17) and *H pylori* antibodies (HpAb) of both IgG and IgA class from the same sample using the ELISA technique has been validated[28-30]. In addition to corpus atrophy, the GastroPanel examination also allows exploration of the structure and function of the antrum mucosa, and can indicate the presence of intragastric acidity[31-34].

The aim of this study was to examine, in a Japanese population, how well the European GastroPanel examination delineates patients with atrophic gastritis, and, in particular, how well these examinations differentiate between patients with healthy and diseased gastric mucosa. A second aim was to examine how the conventional Japanese Pg assays fit with those in the European GastroPanel examination.

MATERIALS AND METHODS

Patient series

A total of 162 subjects (95 men) with a mean age of 55 years (range, 22-79 years) who visited the Tohoku University outpatient clinic for upper GI endoscopy were prospectively enrolled in this study from July 2006 to January 2008. The reasons for endoscopic examination were as follow: dyspeptic symptoms in 42 subjects, screening purposes in 54 asymptomatic subjects, annual endoscopic check-up in 38, and positive results during mass screening with barium meal examinations in 28. When enrolling the participants, individuals with a history of gastric surgery, prior *H pylori* eradication therapy, serious systemic disease, and those taking anti-secretory or anti-coagulant drugs were excluded. A fasting blood sample was obtained from each patient before endoscopy, and the serum was separated and stored in a dichotomous fashion at -20℃. An aliquot of each serum sample was subjected to both Pg assay using the Japanese technique and the GastroPanel examination as described below.

Endoscopy and biopsy

Diagnostic upper GI endoscopy was performed in all patients. Endoscopic examination revealed duodenal ulcer scar in 11 subjects, gastric ulcer or gastric ulcer scar in 10, reflux eosophagitis in six, duodenal adenoma in one, gastric adenoma in one, and no abnormal findings or gastritis alone in the others. Endoscopic biopsies were taken from the antrum and corpus, all along the greater curvature (one biopsy from both sites). Biopsy specimens were routinely fixed in neutral formalin and processed in paraffin. Tissue sections were stained with HE, Alcian blue and modified Giemsa (*H pylori* stain) methods.

Classification of patients

Based on histological appearances of the antral and corpus biopsies, the patients were classified into five categories. These categories were:

Atrophic gastritis in corpus alone (C): moderate or severe atrophy (40%-100% loss of normal oxyntic glands with the appearance of intestinal metaplasia and chronic inflammation in varying degree in the available corpus biopsy, in association with normal appearance of the antrum biopsy).

Atrophic gastritis in antrum and corpus (AC): moderate or severe atrophy (40%-100% loss of normal oxyntic and antral (pyloric) glands with the appearance of intestinal metaplasia and chronic inflammation in varying degree in the available antral and corpus biopsies).

Atrophic gastritis in antrum alone (A): moderate or severe atrophy (40%-100% loss of normal antral (pyloric) glands with the appearance of intestinal metaplasia and chronic inflammation in varying degree in the available antral biopsy, in association with normal appearance of the corpus biopsy).

Non-atrophic ("superficial") chronic gastritis (S): no atrophic or metaplastic changes, but the presence of chronic inflammation of varying degree and activity, and with varying grades of *H pylori* in the antrum and/or corpus biopsies.

Normal stomach mucosa (N): mucosa normal in both antrum and corpus biopsy. No atrophy, metaplasia or inflammation.

Categories C, AC and A represented patients with advanced (moderate or severe) atrophic gastritis (AG). Category N represented patients with healthy and normal stomach mucosa. The category of patients with "diseased" gastric mucosa included all those in categories S, A, AC and C.

The biopsy specimens were interpreted by an experienced pathologist (Professor Pentti Sipponen,

Helsinki University Hospital, Helsinki, Finland) without knowledge of the clinical data or results from the biomarker analyses.

Pg Ⅰ and Ⅱ assays with the Japanese technique

Serum levels of Pg were measured by chemiluminescent enzyme immunoassay using commercial kits (Lumipulse pepsinogen Ⅰ & Ⅱ, Fujirebio Inc., Tokyo, Japan)[35]. For the diagnosis of atrophic corpus gastritis, three different criteria were used as follows[22,36,37]: "Mild" criteria: Pg Ⅰ ≤ 70 μg/L and Pg Ⅰ/Ⅱ ≤ 3.0; "Moderate" criteria: Pg Ⅰ ≤ 50 μg/L and Pg Ⅰ/Ⅱ ≤ 3.0; "Strict" criteria: Pg Ⅰ ≤ μg/L and Pg Ⅰ/Ⅱ ≤ 2.0; For each group of criteria, both cut-offs for Pg Ⅰ and Pg Ⅰ/Ⅱ were required to be fulfilled at the same time.

GastroPanel examination

Pg Ⅰ and Ⅱ, amidated gastrin-17, and IgG and IgA class antibodies to *H pylori* were determined using specific ELISA tests (Biohit Plc, Helsinki, Finland) and were performed in batches of 40 samples on a micro-well plate, according to the manufacturer's instructions. All EIA techniques were based on the measurement of absorbance after the peroxidation reaction at 450 nm. Between the reaction steps the plates were washed using a BW50 Microplate Strip Washer (Biohit Plc, Helsinki, Finland). Absorbances were measured using a micro-well plate reader (BP800 Microplate Reader, Biohit Plc, Helsinki, Finland). To determine PgI and gastrin-17 values, second order fits on standard concentrations were used to interpolate/extrapolate from unknown sample concentrations automatically with the help of the BP800 in-built software (Biohit Plc, Helsinki, Finland).

H pylori antibodies were expressed as enzyme immuno units (EIU) according to the formula included in the test kit: Sample EIU = [X (A_{Sample})-X (A_{Blank})]/[X ($A_{Calibrator}$)-X (A_{Blank})]. EIU levels ≥ 30 were considered *H pylori* positive. In the GastroPanel examination, normal ranges for serum/plasma Pg Ⅰ, Pg Ⅱ, Pg Ⅰ/Ⅱ ratio and amidated gastrin-17 were determined by the manufacturer as 30-165 micro/L, 3-15 micro/L, 3-20, and 1-10 pool/L, respectively (www.gastropanel.net).

According to available validations of the GastroPanel examination against endoscopic histology, advanced (moderate or severe) atrophic gastritis was observed with high accuracy ("strict" criteria) if the serum/plasma Pg Ⅰ was < 30 μg/L and/or Pg Ⅰ/Pg Ⅱ ratio < 3[28,29]. Advanced (moderate or severe) antral atrophy or antral predominant *H pylori* gastritis was observed if the HpAb test was positive and fasting serum G-17 < 1 pmol/L.

Classification of patients into different gastritis categories by the GastroPanel examination

Classification of patients using the GastroPanel examination into categories C, AC, A, S or N was carried out using cut-offs for the test parameters as provided by the manufacturer and by using the GastroSoft® computer program (Biohit Plc, Helsinki, Finland). This computer program is based on extensive background material obtained endoscopically and histologically, the program calculates the probabilities for all diagnostic categories from this database. Finally, the GastroSoft program automatically provides the most likely alternative diagnosis. Classification of patients using the GastroPanel examination was done without knowledge of endoscopy and histology results.

Statistical analysis

For the GastroPanel examination and the conventional Japanese Pg assay, the accuracy, sensitivity, and specificity were estimated and compared with histological assessment of the antrum and corpus biopsies. These statistical parameters for the diagnosis of atrophic gastritis were calculated from the serological tests to discriminate histological C, AC, and A from S and N. In the differentiation analysis between patients with healthy and diseased stomach mucosa, these parameters were calculated and used to discriminate C, AC, A, and S from N. The correlations in serum Pg levels between the GastroPanel examination and the conventional Japanese assay were assessed using linear regression analysis, and Pearson correlation coefficients (r) were estimated for each analysis. The study was approved by Tohoku University School of Medicine Ethics Committee and each subject gave written informed consent.

RESULTS

Serum levels of Pg

Serum levels of Pg Ⅰ and Pg Ⅱ correlated significantly and very well ($r = 0.97$, $P < 0.001$ and $r = 0.98$, $P < 0.001$, respectively) between the Japanese assays and the GastroPanel methods in the same serum samples (Figure 1A and B). A technical and methodological difference did exist, however, in that the Pg Ⅰ test in the GastroPanel examination gave exactly twice the Pg Ⅰ level to that in the Japanese assays. No differences were observed between the Pg Ⅱ tests. Accordingly, the Pg Ⅰ/Pg Ⅱ ratio in the GastroPanel examination was exactly twice the ratio in the conventional Japanese tests, even though the correlation between the Pg ratios was highly significant and very good (Figure 1C; $r = 0.96$, $P < 0.001$).

Atrophic gastritis

GastroPanel: Using the "strict" criteria for advanced atrophic gastritis (moderate or severe in grade; see Materials and Methods), Table 1 shows the distribution of patients into the different gastritis categories. When compared to endoscopic histology, the accuracy of the GastroPanel examination to diagnose atrophic gastritis was 87%, the sensitivity was 40% and the specificity 94%.

Conventional Japanese Pg assay: Using the "strict" criteria for cut-offs of Pg levels (Pg Ⅰ ≤ 30 μg/L and Pg Ⅰ/Ⅱ ≤ 2.0) in the Japanese assay, Table 2 shows the distribution of patients into positive and negative groups regarding atrophic gastritis. When compared to endoscopic histology, the accuracy, sensitivity and specificity of the test were 88%, 45% and 96%,

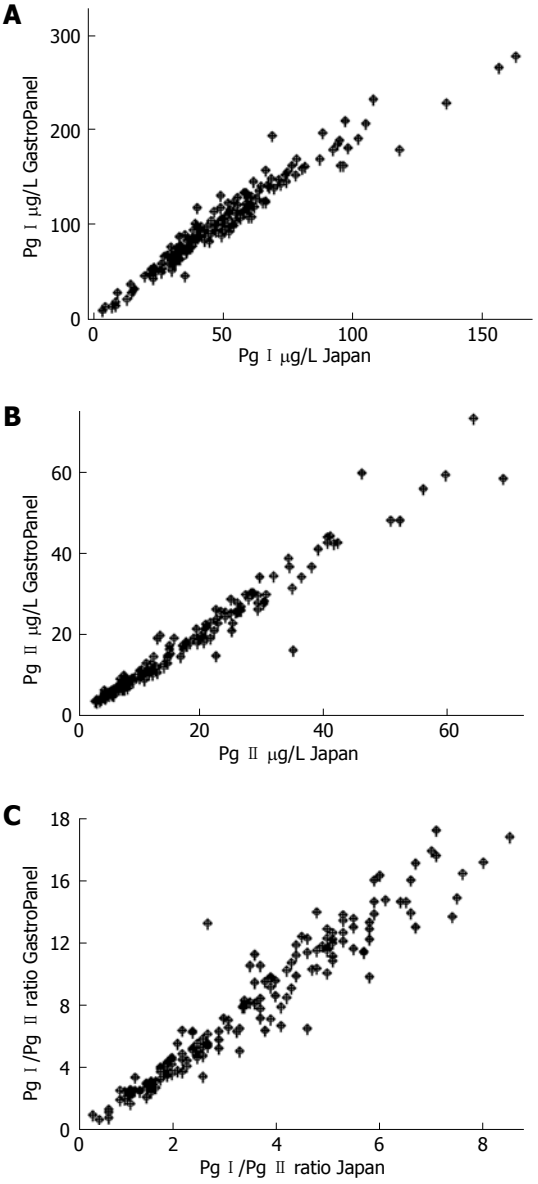

Figure 1 Serum levels of Pg using the GastroPanel examination and the Japanese Pg test in the same serum samples. A: Serum Pg Ⅰ; B: Serum Pg Ⅱ; C: Serum Pg Ⅰ/Ⅱ ratio.

respectively, which corresponded well with those from the GastroPanel examination. If the pepsinogen criteria were lowered to "moderate" (Pg Ⅰ ≤ 50 μg/L and Pg Ⅰ/Ⅱ ≤ 3.0) or "mild" (Pg Ⅰ ≤ 30 μg/L and Pg Ⅰ/Ⅱ ≤ 2.0), the sensitivity of the examination increased but the specificity decreased (Table 3).

Differentiation between patients with healthy and diseased stomach mucosa

The GastroPanel examination, but not Pg testing alone, enabled the differentiation of patients into those with healthy or diseased mucosa (presence of *H pylori* gastritis or atrophic gastritis which may be *H pylori* positive or negative). The GastroPanel test included assays of Pg Ⅰ and Pg Ⅱ as biomarkers for atrophic gastritis in corpus mucosa but also included an amidated G-17 assay as a biomarker of structure and function of the gastric antrum, and assays for the presence or absence of *H pylori* antibodies as a biomarker of on-going *Helicobacter* infection and gastritis. If all biomarkers in the GastroPanel examination were normal, the stomach mucosa was considered normal and healthy. If any of the biomarkers were abnormal, the patient was considered to have *H pylori* gastritis or atrophic gastritis. Using this delineation (see Materials and Methods), Table 4 shows the distribution of the patients into two subgroups (i.e. those with healthy and normal stomach versus those with *H pylori* gastritis or atrophic gastritis). In this setting, the findings from biopsy histology were compared between the two delineated subgroups. In this analysis, the accuracy, sensitivity and specificity of the GastroPanel test to diagnose healthy stomach mucosa were 94%, 95% and 93%, respectively.

Table 1 Prevalence of patients in different gastritis categories. Comparison between GastroPanel examination and biopsy histology

GastroPanel	Histology					
	C	AC	A	S	N	Total
C	3	0	0	4	0	7
AC	1	2	0	0	0	3
A	0	1	1	5	0	7
S	3	1	5	73	3	85
N	0	2	1	4	53	60
Total	7	6	7	86	56	162

Accuracy: 87%; sensitivity: 40%; specificity: 94%; C, AC, A: Moderate or severe atrophic gastritis in corpus alone, in antrum and corpus simultaneously, and in antrum alone, respectively; S: Non-atrophic *H pylori* gastritis; N: Normal and healthy stomach mucosa.

Table 2 Prevalence of patients in corpus atrophy positive (AG+) and negative (AG-) categories if "strict" criteria for cut-off of positive Pg test (Pg test+ versus Pg test-) are used. Comparison between Japanese Pepsinogen test and biopsy histology

	AG+	AG-	Total
Pg test+	9	6	15
Pg test-	11	136	147
Total	20	142	162

AG: Atrophic corpus gastritis present (+) or absent (-). Pg: Pepsinogen test positive (+) or negative (-) for atrophic corpus gastritis.

Table 3 Sensitivity and specificity of the Japanese pepsinogen test and GastroPanel examination in atrophic gastritis if the cut-offs (criteria) for the positive pepsinogen test result are set to be mild, moderate or strict (%)

Pepsinogen test criteria	Sensitivity	Specificity
Japanese-mild	75	69
Japanese-moderate	65	77
Japanese-strict	45	96
Gastropanel-strict	40	94

DISCUSSION

The present analysis showed that non-invasive serum Pg assays accurately diagnosed Japanese patients with atrophic corpus gastritis. Similar findings were also obtained

Table 4 Prevalence of patients in categories of "healthy" or "diseased" gastric mucosa. Comparison between GastroPanel examination and biopsy histology

GastroPanel	Histology		
	Healthy stomach mucosa	Diseased stomach mucosa	Total
Healthy stomach mucosa	53	7	60
Diseased stomach mucosa	3	99	102
Total	56	106	162

Accuracy: 94%; sensitivity: 95%; specificity: 93%.

using both the conventional Japanese Pg tests and the Pg assays of the novel European GastroPanel examination in which, in addition to Pg, the serum/plasma levels of amidated gastrin-17 (G-17) and *H pylori* antibodies (IgG and IgA) were also measured. The diagnostic accuracy of both the Japanese test and the GastroPanel test was more than 80% when compared with endoscopic biopsy histology. In addition, it is noteworthy that, since both the Japanese and the European (GastroPanel) Pg tests seemed to fit without any exceptions, no racial differences could be demonstrated in Pg antigens by the present study-both the Japanese and European assays gave very similar results.

The GastroPanel test included assays of amidated G-17 and *H pylori* antibodies in addition to the Pg assays. The rationale for this is that the serum level of amidated G-17 is a biomarker of the function and structure of the gastric antral mucosa. Serum levels of G-17 were high in subjects with atrophic gastritis limited to corpus mucosa alone but normal and low in those in whom atrophic gastritis was present in both the antrum and corpus (multifocal atrophic gastritis of Correa - highest risk condition for gastric cancer known so far). The rationale for the serological *H pylori* test, on the other hand, is that the presence or absence of *H pylori* antibodies in serum is the most reliable biomarker of an on-going *H pylori* infection. When compared with the ^{13}C urea breath-test (UBT) or stool antigen test, the serological test avoids false-negative results which appear in more than half of patients with atrophic corpus gastritis (hypochlorhydric stomach) or PPI use when analyzed using the UBT or stool antigen test. In this sense, the GastroPanel biomarker examination provides a most reliable tool for delineating between patients with healthy stomach and those with *H pylori* non-atrophic gastritis or atrophic gastritis.

In the present study, the biomarker tests were compared with endoscopic biopsy histology. Endoscopic biopsy histology is, however, not a reliable gold standard. Biopsy results are commonly biased by several factors, including such confounders as biopsy sampling, number of biopsies available from each stomach compartment, laboratory processing of the specimens, and interpretation of the biopsy by pathologists. In the present study, the biopsy analysis was based on only one biopsy from both the antrum and corpus, and so the study protocol did not strictly follow the guidelines of the Sydney System (the guidelines indicate at least two biopsies from each compartment). Interpretation of the biopsy findings by pathologists may, therefore, easily fail, particularly in antral biopsies, in which the interobserver agreement, even between "expert" pathologists, is known to be imperfect and may require practice or even the application of morphometry[38-40].

Biomarker examinations from serum or plasma are free of the biases that affect biopsy histology or sampling. The biomarkers give an average view of the structure and function of the stomach mucosa. In addition to the Pg tests, the GastroPanel examination included assays of serum/plasma levels of amidated G-17 and *H pylori* antibodies. This also allows insight into the function and structure of the gastric antrum, confirms Pg assays, and can suggest the presence of intragastric acidity[31-34]. A low fasting level of serum/plasma G-17 indicates subjects with high intragastric acidity (acid inhibits the release of amidated G-17 from antral G cells) or those with atrophy of the antral mucosa (the loss of antral glands also results in loss and disappearance of antral G cells)[28,29]. In the present study population, seven patients were classified into this category according to the GastroPanel examination. These seven patients were anticipated to have an antrum-limited atrophic gastritis or *H pylori* gastritis that was strongly antrum predominant (a phenotype of *H pylori* gastritis that is associated with the risk of peptic ulcer disease (PU), particularly PU of the duodenal ulcer type)[10]. Low fasting levels of serum/plasma G-17 in connection with low Pg I or Pg I /Pg Ⅱ ratio also identifies subjects who have the highest known risk of gastric cancer; i.e. patients with advanced and extensive atrophic gastritis in both the antrum and corpus (advanced multifocal atrophic gastritis)[4,6,17]. In the present study, three patients (2%) were classified into this category using the GastroPanel examination, which was also confirmed by biopsy histology.

Differentiation between patients with healthy and diseased gastric mucosa is one of the key issues in assessing the risks for serious gastric diseases in clinical practice. If the stomach mucosa is healthy, the risks of serious gastric diseases (cancer or peptic ulcer) are extremely low (nil in practice). With high certainty (accuracy 94%, sensitivity 95% and specificity 93%) the GastroPanel examination indicated that 53 of 162 patients (33%) in this study had normal and healthy stomach mucosa.

Biomarker tests are not "cancer tests". However, they can be used in the screening and diagnosis of subjects with a high cancer risk; i.e. subjects with atrophic gastritis in which a careful diagnostic endoscopy (gastroscopy) is mandatory to find possible neoplastic or precancerous lesions at an early and curable stage. In the post hoc analysis, none of these 53 patients with "healthy" stomach had neoplastic lesions or signs of active peptic ulcers on endoscopy (one patient had a duodenal scar and one had a scar in the stomach mucosa). On the other hand, two of the patients with atrophic gastritis had neoplastic gastric or duodenal adenoma. Thus, in the present study population, all neoplastic gastroduodenal

lesions were found in those patients with diseased stomach mucosa using the GastroPanel examination.

The reasons for the differences in serum levels of Pg I between the Japanese and GastroPanel assays are technical and methodological, and are most likely due to differences in the calibrators used in the assay technique. However, due to the excellent correlations between the tests, the results from the conventional Japanese Pg I assay can easily be converted (by doubling the test results) to correspond with those obtained using the GastroPanel Pg I test, or *vice versa*.

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COMMENTS

Background

Reliable non-invasive diagnosis of *Helicobacter pylori (H pylori)* gastritis and atrophic gastritis, and the delineation of patients with healthy stomach mucosa, are clinically important tasks.

Research frontiers

Biomarker tests are potential non-invasive diagnostic tools for assessment of the function and structure of the stomach mucosa.

Innovations and breakthroughs

Available pepsinogen (Pg) tests, both Japanese and European, are excellent in Asian outpatients when compared in a "head-to-head" analysis in the same study population. The addition of assays for serum amidated gastrin-17 and serological *H pylori* tests to the Pg assays increases the clinical applicability of the biomarker tests.

Applications

A comprehensive set of biomarker tests (GastroPanel) is applicable in the reliable diagnosis of *H pylori* gastritis, atrophic gastritis, and also in the delineation of subjects with healthy, normal stomach mucosa.

Peer review

The authors evaluated the predictive value of the detection of a set of serum biomarkers (Pg Ⅰ/Pg Ⅱ, gastrin-17, and antibodies against *H pylori*) using the European GastroPanel examination among 162 Japanese patients. They found that the GastroPanel examination classified the patients into groups with "healthy" or "diseased" stomach mucosa with 94% accuracy, 95% sensitivity and 93% specificity, as compared to endoscopic biopsy findings. It is helpful for readers to understand the usefulness of this examination among Asian gastric patients.

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BRIEF ARTICLES

Furazolidone, amoxicillin, bismuth and rabeprazole quadruple rescue therapy for the eradication of *Helicobacter pylori*

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Abstract

AIM: To compare the efficacy and side effect profiles of three furazolidone and amoxicillin-based quadruple rescue therapies for the eradication of *Helicobacter pylori* (*H pylori*).

METHODS: Patients who failed in the *H pylori* eradication therapy for at least one course were randomly allocated into three groups. Group A received rebaprazole 10 mg + amoxicillin 1 g + furazolidone 100 mg, and bismuth subcitrate 220 mg, twice daily for 1 wk; group B received the same regimen of group A but for 2 wk; and group C received the same regimen of group B, but furazolidone was replaced by furazolidone 100 mg three times daily. To record the side effect profiles at the end of the treatment, *H pylori* eradication was assessed with ^{13}C-urea breath test 4 wk after therapy.

RESULTS: Sixty patients were enrolled including 28 males, and 20 patients in each group. The average age of the patients was 49.2 years, ranging from 18 to 84 years. *H pylori* eradication rates with per-protocol analysis were 82%, 89% and 90% in the three groups, respectively. Side effects were found in 11 patients, including mild dizziness, nausea, diarrhea and increased bowel movement. None of the 11 patients needed treatment for their side effects.

CONCLUSION: One- or two-week furazolidone and amoxicillin-based quadruple rescue therapy with a low dose furazolidone (100 mg *bid*) for the eradication of *H pylori* is effective. Extending the antibiotic course to 14 d could improve the eradication rates.

Key words: *Helicobacter pylori*; Rescue therapy; Quadruple therapy; Furazolidone; Low dose

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is associated with many upper gastrointestinal diseases, such as chronic gastritis, peptic ulcer, gastric carcinoma and mild malignant mucosa-associated lymphoid tissue lymphoma (MALToma). During recent years, the efficacy of the first-line therapy including proton pump inhibitors plus two antibiotics seems to have decreased, and several studies have reported intention-to-treat eradication rates lower than 75%[1-3] and even lower than 50%[4,5]. The resistance to antibiotics is the main cause of *H pylori* treatment failure. A multicenter study conducted in China in 2005 showed that the resistant rates of *H pylori* were 27.4% for clarithromycin, 75.6% for metronidazole and 2.7% for amoxicillin[6]. Many patients needed to receive rescue therapy for the eradication of *H pylori* after first- or second-line therapies. The combination of metronidazole, tetracycline, bismuth, and proton pump inhibitor are currently considered standard rescue regimens for the treatment of *H pylori* infection[7]. However, metronidazole resistance is a rising problem worldwide, particularly in developing countries, such as China, which limits the usefulness of this drug.

Furazolidone, a monoamine oxidase inhibitor, has broad antibacterial activity based on interference with bacterial enzymes. It has already been used to treat peptic ulcer disease for many years in China, before *H pylori* was discovered[8,9]. Furazolidone emerged as an agent for *H pylori* eradication regimens due to its low cost and prevalence of resistant strains in China.

The consensus of reports of China in 2005[10] and 2007[11] all recommended that furazolidone should be used for *H pylori* eradication treatment. A large multicentrer study in China showed that a combination of omeprazole plus furazolidone and amoxicillin as the first-line regimen had an intention-to-treat *H pylori* eradication rate of 86%, compared to 69% for omeprazole plus clarithromycin and metronidazole[12]. However, in another study in China, the intention-to-treat eradication rate of omeprazole-furazolidone-amoxicillin regimen as the rescue regimen was only 52%[13]. The aim of this pilot study was to compare the efficacy and side effect profiles of three different furazolidone and amoxicillin-based quadruple rescue therapies for the eradication of *H pylori*.

MATERIALS AND METHODS

Subjects

This prospective clinical trial was conducted in Peking University First Hospital. Patients who failed in the eradication of *H pylori* for at least one course were invited to participate in this open-label pilot study. Informed written consent was obtained from all patients participating in the trial.

Patients younger than 18 years of age, and who presented with severe comorbidity, who were pregnant or lactating, with a known history of allergy to the study drugs, and patients who had used proton pump inhibitors, H_2 receptor blockers, antibiotics, or bismuth salts up to 4 wk before the study were all excluded.

Treatment regimen

Patients were randomly allocated into three groups. Group A received rebaprazole 10 mg, amoxicillin 1 g, furazolidone 100 mg, and bismuth subcitrate 220 mg, twice daily each, for 1 wk; group B received the same regimen as group A but for 2 wk; and group C received the same regimen of group B but furazolidone was replaced by furazolidone 100 mg three times daily. Antibiotics were prescribed after meals whereas rabeprazole and bismuth were adminstered before meals. Patients were advised to maintain the treatment even with minor adverse effects. No other medication was allowed until the end of the treatment.

Assessment

Patients were evaluated using the ^{13}C-urea breath test at least 4 wk after *H pylori* eradication treatment. Antimicrobials, bismuth-containing drugs and acid-reducing agents were not allowed during the 4 wk preceding the ^{13}C-urea breath test. The eradication of *H pylori* was defined as a negative urea breath test.

The patient compliance and treatment-related side effects were assessed at the end of the treatment. Side effects were graded as mild if they did not interfere with daily activities of the patients, moderate if they interfered with daily activities to some extent and severe if daily activities became impossible.

Statistical analysis

Continuous variables were expressed by calculation of the mean and standard deviation. The *H pylori* eradication rate was assessed based on intention-to-treat and per-protocol analysis. The 95% confidence intervals (95% CI) were also calculated for both intention-to-treat and per protocol analysis and the eradication rate. The patients, who were lost to follow-up or could not complete the treatment course because of severe side effects, were considered as treatment failures and excluded in the per-protocol analysis. The Chi-square test and Fisher's exact test were used to compare the differences between the three study groups in terms of baseline data, eradication rate and side effects. $P < 0.05$ was considered significant.

RESULTS

Sixty patients were enrolled in this study including 28 males, with 20 patients in each group. All of the patients had undergone endoscopy examination before they received *H pylori* eradication at the first time. The average age of the patients was 49.1 years, ranging from 18 to 84 years. Two patients had already undergone three treatments, 42 and 16 had undergone one and two, respectively. There was no predominance regarding the baseline characteristics of the patients (Table 1).

All the patients finished the treatment, but four of them did not receive the ^{13}C-urea breath test examination. *H pylori* eradication rates with per-protocol analyses were 82%, 89% and 90% in the three groups, respectively ($P > 0.05$) and the intention-to-treat eradication rates were 70% (14/20), 85% (17/20) and 90% (18/20) in the three groups ($P > 0.05$) (Table 2). No significant difference was found between the eradication rates of the patients who failed *H pylori* eradication for one, two or three courses (Table 3).

Side effects were found in 11 patients, including mild dizziness, nausea, diarrhea and bowel movement increase (Table 4). None of the 11 patients needed treatment or stopped the therapy for their side effects.

DISCUSSION

The eradication of *H pylori* is the main objective in the treatment of peptic ulcer[14,15]. The Masstricht Ⅲ consensus report concluded that eradication of *H pylori* has the potential to reduce the risk of gastric cancer development; moreover, the optimal time to eradicate *H pylori* is before pre-neoplastic lesions (atrophy and intestinal metaplasia) are present[7]. Gastric carcinoma is common in China. So *H pylori* infection is a major public health problem, for which treatment should be provided when patients are diagnosed. The ideal therapy for *H pylori* infection should achieve a high cure rate of > 90% on per protocol analysis and > 80% on intention-to-treat analysis, should be simple and well tolerated, and should be easy to comply with and cost-effective[16]. The combination of proton pump inhibitor plus bismuth, tetracycline and metronidazole has been

Table 1 Baseline characteristics of patients (mean ± SD)

Group	No. of patients	Age (yr)	Male	Gastritis	Ulcer (DU/GU)	Smoking	Drinking
A	20	49.3 ± 15.2	8	11	8 (7/1)	6	3
B	20	49.9 ± 21.5	12	13	9 (8/1)	6	5
C	20	48.1 ± 9.3	8	12	7 (7/0)	6	3
Total	60	49.1 ± 15.8	28	36	24	18	9
χ^2 value	-	-	2.14	0.42	0.42	0.00	0.89
P value	-	-	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Table 2 *H pylori* eradication rates among three treatment groups

Group	Eradication	Non-eradication	Lost	Total	Eradication rate[a] (PP, %) (95% CI, %)	Eradication rate[b] (ITT, %) (95% CI, %)
A	14	3	3	20	82 (57-96)	70 (46-88)
B	17	2	1	20	89 (67-99)	85 (62-97)
C	18	2	0	20	90 (68-99)	90 (68-99)
Total	48	7	4	60	87 (78-96)	80 (70-90)

[a]$\chi^2 = 0.59$, $P > 0.05$; [b]$\chi^2 = 2.89$, $P > 0.05$.

Table 3 *H pylori* eradication rates in relation to course of previous treatment

Times of failure	Eradication	Non-eradication	Lost	Total	Eradication rate[a] (PP,%) (95% CI, %)	Eradication rate[b] (ITT, %) (95% CI, %)
1	35	5	2	42	88 (73-96)	83 (74-96)
2	13	1	2	16	93 (66-100)	81 (54-96)
3	1	1	0	2	50 (1-99)	50 (1-99)
Total	48	7	4	60	87 (78-96)	80 (70-90)

[a]$\chi^2 = 2.94$, $P > 0.05$; [b]$\chi^2 = 1.42$, $P > 0.05$.

Table 4 Side-effect profile of patients *n* (%)

Side effects	Group A	Group B	Group C	Total
Nausea	2	1	2	5 (8.3)
Diarrhea	1	1	1	3 (5.0)
Bowel movement increasing	0	1	1	2 (3.3)
Dizziness	0	0	2	2 (3.3)
Headache	0	0	1	1 (1.7)
Asthenia	1	0	0	1 (1.7)
Total episodes	4	3	7	14 (23.3)
Total patients[1]	2 (10)	3 (15)	6 (30)	11 (18.3)

Two patients got two kinds of side effects in group A and group C, respectively; [1]$\chi^2 = 2.89$, $P > 0.05$.

recommended as optimal second-line therapy by several guidelines on the management of *H pylori* infection[7]. However, metronidazole resistance is largely responsible for treatment failure. The prevalence of metronidazole resistance for *H pylori* is about 80% in China[6]. The best rescue treatment remains to be defined.

Furazolidone is a broad-spectrum nitrofuran, active against Gram-negative and positive bacteria and protozoa by inhibiting bacterial enzymes, and it has poor oral absorption[17]. Strains resistant to furazolidone are rare and have no cross-resistance to metronidazole[18]. Furthermore, its potential to develop resistance is low[19]. Several studies have shown the efficacy of regimens containing a high-dose furazolidone (200 mg, *b.i.d.*) as the therapy in patients with *H pylori* infection[20-22]. The study of Fakheri *et al* showed that low-dose furazolidone (100 mg, *b.i.d.*) based triple and quadruple rescue regimens do not yield acceptable success rates[23]. We have reported that a pilot study of rabeprazole, bismuth, furazolidone and amoxicillin as rescue treatment of *H pylori* infection after failure for at least one course of eradication has intention-to-treat and per-protocol eradication rates of 70% and 82% for 1 wk in the group treated with furazolidone (100 mg, *b.i.d.*), 85% and 89% for 2 wk in the group with furazolidone (100 mg, *b.i.d.*), and 90% and 90% for 2 wk in the group with furozolidone (100 mg, *t.i.d.*), respectively. Our study is different from Fakheri *et al*, as a regimen which is useful in one area may not be effective in another area.

Management of first- or second-line *H pylori* eradication failures has become a challenge. In one study, a *H pylori* eradication rate of 69% was obtained after treatment with a 7-d association of bismuth, high-dose furazolidone (200 mg, *b.i.d.*), amoxicillin and a

proton-pump inhibitor for patients with peptic ulcer who failed to respond to other eradication regimens[24]. In another study, a similar eradication rate (63%, intention-to-treat) was achieved with a rescue treatment of a 7-d quadruple regimen with omeprazole, bismuth, tetracycline, and high-dose furazolidone (200 mg, *b.i.d.*)[25]. Currently, a standard third-line therapy is lacking, and several guidelines recommend a culture to select proper treatment according to microbial sensitivity to antibiotics for these patients[7,11]. However, cultures are often carried out only in research centers. It is a common practice to select a rescue therapy according to experience, especially in China. In this study, the intention-to-treat eradication rates were 73%, 81% and 50% for the patients who failed in *H pylori* eradication for one, two or three courses, respectively. Although, without information of microbial sensitivity to antibiotics, it has been shown that furazolidone-(100 mg, *b.i.d.* or *t.i.d.*) and amoxicillin-based quadruple rescue therapy was highly effective in the population of this region. *H pylori* eradication can be achieved in most patients, even when antibiotic susceptibility is not tested.

Furazolidone presents some side effects, especially gastrointestinal ones[17]. Several studies showed that side effects were very common (more than 20%) in the patients who received treatment with furazolidone-based regimens[13], especially with high-dose furazolidone of 200 mg *b.i.d.*[24,26]. A major problem with furazolidone at high doses is the high rate of severe side effects. Most of these effects are related to its role as a monoamino-oxidase inhibitor and include fever, rash and severe abdominal pain. Such side effects may lead to the discontinuation of treatment in some patients[21]. In this study, the occurrence of side events was 18.3%, and no intolerable side effects leading to early discontinuation of treatment were found. The most common side effects were nausea and diarrhea. Although the side effects were more frequent after extending the treatment course and adding furazolidone, this difference was not significant among different treatment groups.

The weakness of this study is that although set up as a controlled trial, the number of patients in each arm was small. Further studies should be done to conclude whether the increased dose of furazolidone or the longer period of treatment is helpful.

In conclusion, our study shows that the association of rabeprazole, bismuth, amoxicillin, and low-dose furazolidone is a valuable rescue treatment for patients who failed to respond to the first- or second-line *H pylori* eradication in China. Lower doses of furazolidone could decrease the incidence of side effects, but this strategy can also lead to a lower eradication rate. However, extending the antibiotic course to 14 d could improve eradication rates, despite a greater likelihood of side effects. The regimens are well tolerated by most patients. These are effective, cheap and safe options for salvage therapy of *H pylori* positive patients, and may be recommended as good alternative choice regimens in the eradication of *H pylori* in the population with high metronidazole resistance.

COMMENTS

Background

Helicobacter pylori (*H pylori*) infection is associated with many upper gastrointestinal diseases. The prevalence of *H pylori* resistant to antibiotics was increased with the spreading of *H pylori* eradication. Many patients needed to receive rescue therapy for the eradication of *H pylori* after first- or second-line therapies.

Research frontiers

Furazolidone-based regimens for the eradication of *H pylori* are low in cost. Lower doses of furazolidone could decrease the incidence of side effects. Not many studies have been performed to evaluate the efficacy of low-dose furazolidone-based quadruple regimens for treatment of *H pylori* infection.

Innovations and breakthroughs

This study provides further evidence of the efficacy and tolerability of low-dose furazolidone-based quadruple regimens in China.

Applications

Low-dose furazolidone-based quadruple regimens may be useful rescue therapies for *H pylori* eradication due to their low cost, low resistance rate and relatively minor side effects, especially in developing countries such as China.

Peer review

Furazolidone has been used for the eradication of *H pylori* for many years, but here it has been used as a component of quadruple rescue therapy. This has been reported less frequently. In this series of three groups of 20 patients, in which *H pylori* eradication therapy had failed to respond to at least one previous treatment regimen, each group was treated with one of three regimens of quadruple therapy containing furazolidone (either in different doses or for a different time), and the results were highly successful. Side effects from furazolidone are the main disadvantage of this drug, but when used in small doses as in this study, side effects were relatively minor. For the above reasons this is a useful publication.

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Diagnostic model of saliva protein finger print analysis of patients with gastric cancer

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Abstract

AIM: To explore the method for early diagnosis of gastric cancer by screening the expression spectrum of saliva protein in gastric cancer patients using mass spectrometry for proteomics.

METHODS: Proportional peptide mass fingerprints were obtained by analysis based on proteomics matrix-assisted laser desorption ionization time-of-flight/mass spectrometry. A diagnosis model was established using weak cation exchange magnetic beads to test saliva specimens from gastric cancer patients and healthy subjects.

RESULTS: Significant differences were observed in the mass to charge ratio (m/z) peaks of four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) between gastric cancer patients and healthy subjects.

CONCLUSION: The finger print mass spectrum of saliva protein in patients with gastric cancer can be established using gastric cancer proteomics. A diagnostic model for distinguishing protein expression mass spectra of gastric cancer from non-gastric-cancer saliva can be established according to the different expression of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da. The method for early diagnosis of gastric cancer is of certain value for screening special biological markers.

Key words: Saliva; Protein finger print model; Gastric cancer; Matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry; Weak cation exchange; Magnetic bead; Proteomics

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INTRODUCTION

Gastric cancer is one of the most common cancers. Even though its mortality rate has decreased in recent years, its morbidity still ranks first among all kinds of malignant tumor. Moreover, gastric cancer obviously occurs in juvenescence, and is diagnosed at its advanced stage. About 150-200 thousand patients die of gastric cancer every year in our country, which accounts for almost a quarter of all deaths from malignant tumors. About 200 000 new gastric cancer patients are diagnosed each year. At present, available tumor markers have a relatively high false-positive rate for the diagnosis of gastric cancer, and thus cannot predict early gastric cancer[1,2]. Therefore, it has become important to find a special way to predict early stage gastric cancer.

Saliva can be used in diagnosing gastric cancer, and it has been verified for many years that detection of salivary components is a valuable tool to diagnose a variety of diseases[3]. Salivaas, a diagnostic specimen has received attention. Along with the wide application of proteomics and its related techniques, proteomics has been used more widely in the study of saliva[4,5]. In this study, we employed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technique to analyze the protein mass peak of

saliva from gastric cancer patients and healthy subjects, and to screen for the special biological markers for predicting early gastric cancer.

MATERIALS AND METHODS

Clinical data and specimen selection

Saliva samples were collected from gastric cancer patients in the Medical Department of Nanfang Hospital, Southern Medical University. Control samples were collected from healthy volunteers. Saliva was collected and put into a 15-mL centrifuge tube, and centrifuged at 10000 r/min for 5 min at 4℃. Fifty microliters of each saliva sample was put into in a 1-mL EP tube and stored at -80℃. When experiments were conducted, the samples were taken out from the refrigerator at -80℃. All samples were defrosted at room temperature. The healthy subjects at the age of 23-68 years served as a normal group (n = 18, 12 males, 6 females) and the patients at the age of 25-78 years served as a gastric cancer group (n = 23, 15 males, 8 females).

Instruments and reagents

A weak cation exchange (WCX) magnetic bead kit, alpha-cyano-4-hvdroxycinnamic acid (HCCA), and AutoFlex III MALDI-TOF mass spectrometer were purchased from Bruker Company. Mass concentration (0.3 g/L) and ethanol (chromatographic grade)/acetone (chromatographic grade) = 2/1 were freshly prepared.

Treatment with WCX magnetic beads

The WCX magnetic bead kit was taken out from a refrigerator at 4℃, washed and eluted. Finally, the separated magnetic beads and eluted polypeptide samples were transferred into a 0.5-mL clean sample tube for further MS analysis.

Sample application and MS analysis

One microliter polypeptide sample separated with the magnetic beads was applied. After the polypeptide sample was dried at room temperature, into which 1 μL HCCA substrate solution (3 g/L, dissolved in 50% acetonitrile and 2% trifluoroacetic acid) was added. Then, the prepared sample was applied and analyzed on MALDI-TOF-MS. A linear mode was used to collect peptides with a molecular weight of 1000-10000. Twenty percent of laser energy was used with 400 shots. Peptide mass finger prints were obtained by accumulating 50 single scanning of MS signals.

Statistic analysis

FlexAnalysis 3.0 and ClinProTools 2.1 (from Bruker Company) were used to analyze the grouping index and its dependability of data. $P < 0.05$ was considered statistically significant.

RESULTS

The mass spectrum of samples from 41 subjects was analyzed and compared. Seventy-four protein mass peaks were found. Mass peaks were proved to be significantly different in four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) (Figures 1-4). The protein mass peak at 1472.78 Da was higher in the gastric cancer group than in the normal group. Based on the identification of these four protein mass peaks, 22 patients were accurately diagnosed with gastric cancer. All the 18 healthy subjects were confirmed to have no gastric cancer. The present method for the diagnosis of gastric cancer had a sensitivity of 95.65% (22/23), and a specificity of 100% (18/18). The results are shown in Table 1.

Table 1 Identification of gastric cancer in different groups

Groups	Classification of proteome mass spectrum model			
	n	Gastric cancer	Non-gastric cancer	Ratio (%)
Gastric cancer group	23	22	1	95.65
Normal group	18	0	18	100

DISCUSSION

Saliva is an important and necessary body fluid. Blood constituents such as hormones, amino acids, electrolyte, immunoglobulin and creatinine, can enter saliva through the blood barriers of the capillary walls. As one of the fluids in the human body, variation in saliva constituents is influenced by various pathophysiological changes in the body. Saliva constituents are related to serum and the essential proteins in saliva are positively correlated with serum. For example, there is an extremely good dependability between blood plasma free F and salvia F, which is not influenced by the flow rate and stimulation of saliva. Many proteins in saliva, such as anti-HIV antibody, secretory type leukoprotease inhibitor (SLPI) and IgA, have important physiological functions. SLPI, a kind of single strand polypeptide, can be separated from human parotid gland saliva and only has anti-HIV effects in the oral cavity. Saliva can be atraumatically and conveniently taken, and easily observed at any time. The test results are stable and the sensitivity is rather high, and the tests can be repeated. Since biochemical microanalysis techniques have been significantly improved, saliva can be used as a diagnostic index instead of blood[3,6-9].

Studies on saliva proteomics have received extensive attention worldwide[4,5]. Till now, 309 kinds of protein have been identified in full salivary fluid of healthy subjects, using proteomics techniques, among which, the most important acidic proteins are catheptic enzyme L and hyaluronan-conjugated protein; the most important basic proteins are saliva rich pyrrolidinecarboxylic acid, glycoprotein PRB2, and an unknown protein with a level of 12.8; while the smallest proteins are T cell receptor 8 catenin fragment and hylaxin HNP-3, and the protein with a maximal relative molecular mass is mucoprotein 5B. Of the 309 saliva proteins identified according to

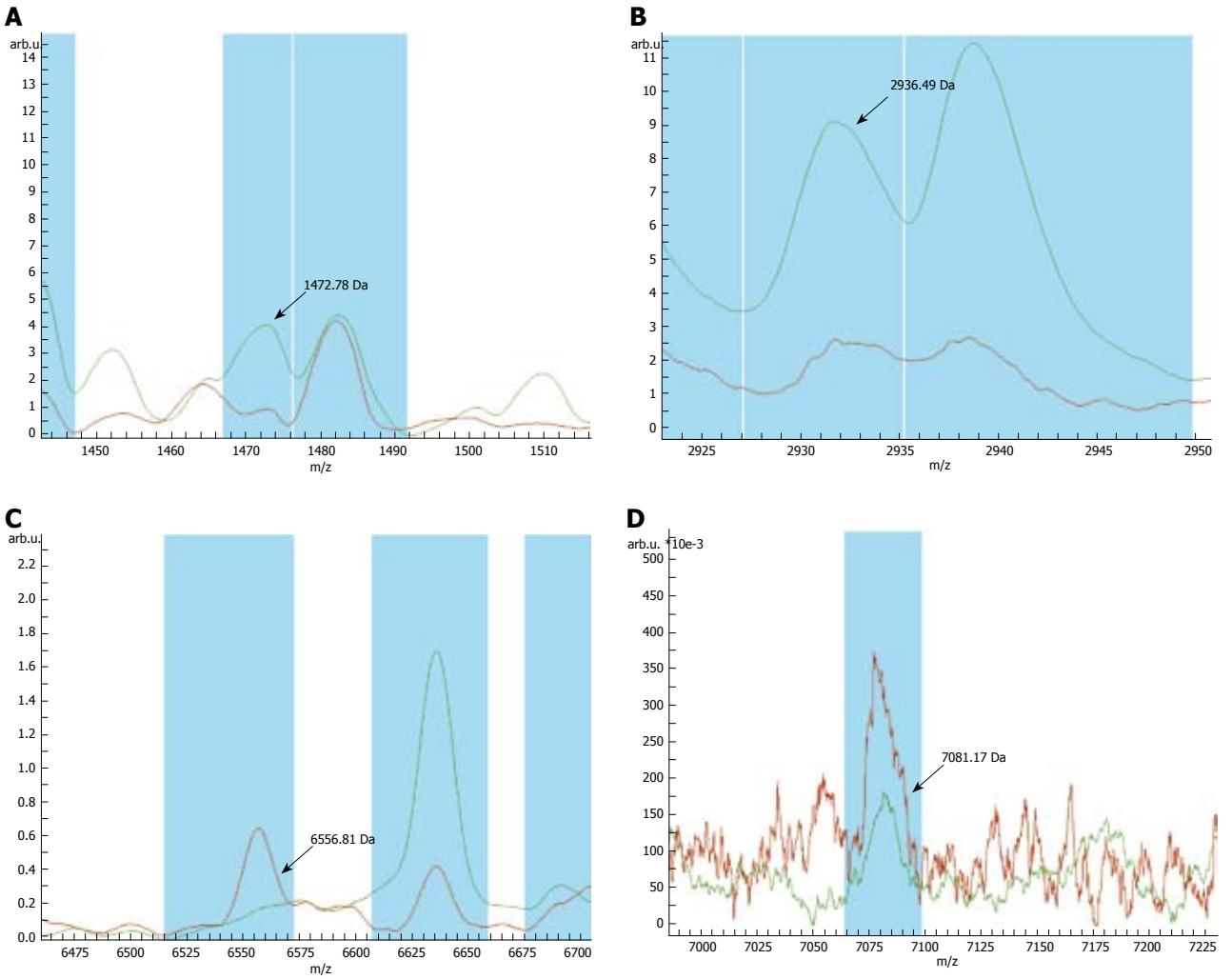

Figure 1 Sample spectrum showing a lower average peak value for proteins 1472.78 Da (A) and 2936.49 Da (B) in the normal group than in the gastric cancer group, and a higher value for proteins 6556.81 Da (C) and 7081.17 Da (D) in the normal group than in the gastric cancer group. Red line: Normal group; Green line: Gastric cancer group.

their functions, 28.7% are uncertain functional proteins, 21% are associated with immunity, 1.6% are associated with protein replication and reparation, 4.8% are associated with cell mobility and secretion, 2.3% are associated with transcription and ribosomes, 4.2% are associated cell multiplication and the cell cycle, 9.7% are associated with signal transduction, 5.2% are associated with metabolism, and 7.1% are associated with the cytoskeleton and endomembrane, respectively. Saliva is an important body fluid with complicated constituents and multiple biological functions. Its distinctive and abundant protein constituents undoubtedly can be used as biological markers of cancer and other diseases. The oral saliva protein flux analysis and precise determination have been restricted by old techniques, since the biological functions of most saliva proteins are unknown, and the value of saliva diagnosis and prognosis remains unclear. With the application of high-flux and high-precision proteomics, it has become possible that saliva proteins can be used as biological markers in prevention and bioprotein-targeted therapy, and in predicting the prognosis of diseases[3-10]. At present, no study on saliva proteomics is available in China, and synchronic detection and comparative study of saliva and serum proteins have not been reported worldwide.

Early stage markers of gastric cancer are a hot spot of investigation around the world. How to find effective and better methods and techniques that can be applied to the treatment of gastric cancer has become the focus of research. There are more than 1000 proteins in saliva, and saliva possesses atraumatic and convenient features, and can be used as a simple and abundant resource. In addition, recent studies have demonstrated that 20% of saliva proteins are similar in the blood, and certain saliva proteins are matched with blood proteins that influence senile dementia, breast cancer and diabetes[10-12]. MS technique is one of the most important techniques in proteomics studies. The most widely used MS technique in proteomics studies is MALDI-TOF MS. Application of MALDI-TOF MS, in study of biological markers of disease, is undoubtedly at the leading edge of molecular diagnosis. Proteomics has become a powerful tool for the discovery of new biological markers. Combining MS techniques and proteomics also provides good prospects for research of biological markers[13,14]. Investigation

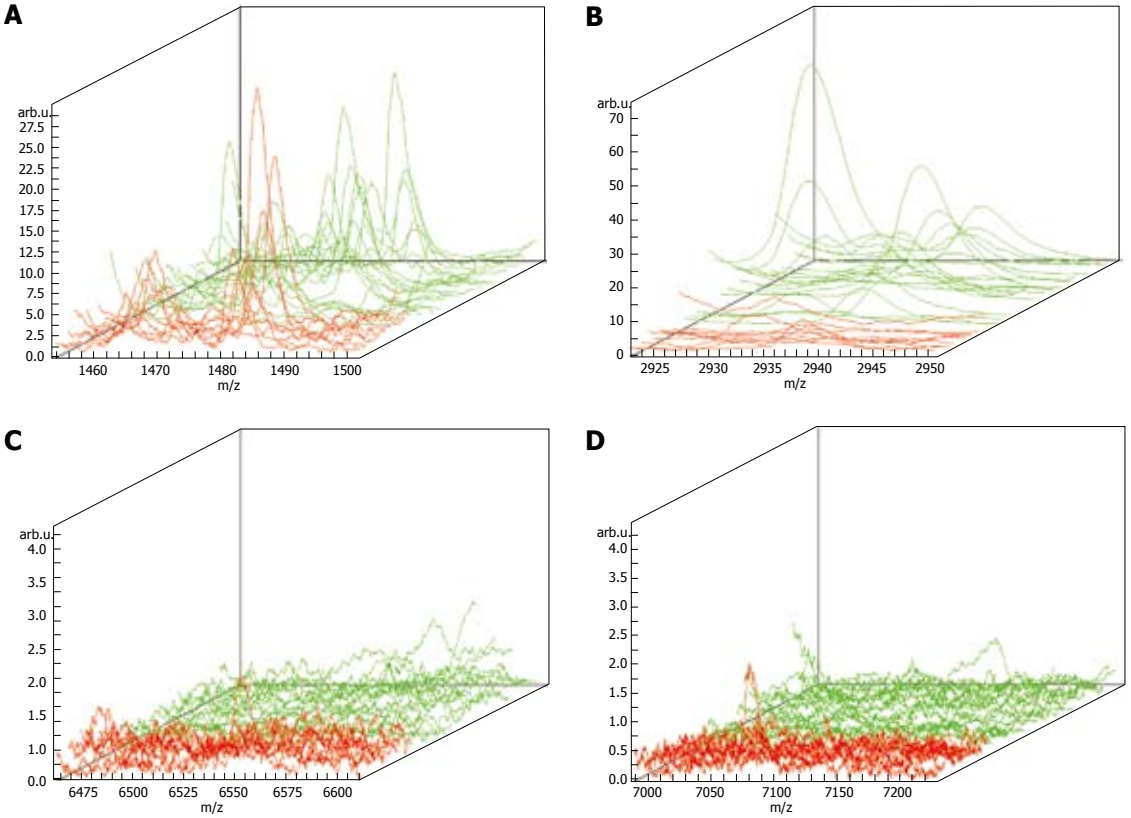

Figure 2 Three-dimensional map showing a lower average peak value for proteins 1472.78 Da (A) and 2936.49 Da (B) in normal group than in gastric cancer group, and a higher value for proteins 6556.81 Da (C) and 7081.17 Da (D) in normal group in gastric cancer group. Red line: Normal group; Green line: Gastric cancer group.

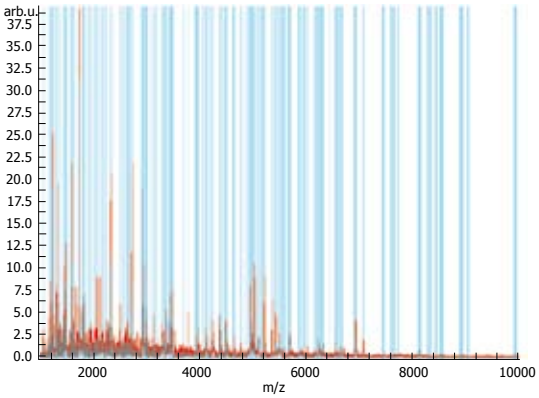

Figure 3 Complete mass spectrum of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da, respectively.

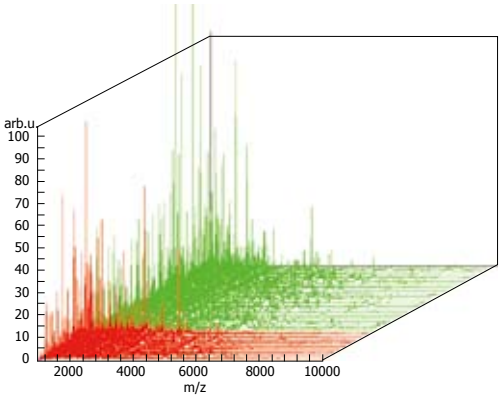

Figure 4 Complete three-dimensional mass spectrum of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da, respectively. Red line: Normal group; Green line: Gastric cancer group.

of proteomics provides not only a material basis for vital movement rules, but also a theoretical basis for overcoming diseases. Certain diseases are related to special protein molecules. Proteins may be the molecular targets of new drugs or molecular markers for the early diagnosis of certain diseases. MS techniques, are important in studies of proteomics, and can be used in the study of protein/peptide spectra, biological marker spectra, or single biological markers for complicated diseases, such as cardiovascular and cerebrovascular disease, tumors, stroke and neuro-degenerative disease. Furthermore, they are also expected to open new avenues for research into pathogenesis, diagnosis and treatment of complicated diseases[15-17].

Gastric cancer usually has a long latent period. Early gastric cancer does not present with overt symptoms. Gastric cancer is at an advanced stage when it is diagnosed and has characteristics such as easy metastasis and poor prognosis. Therefore, early diagnosis is extremely important for the control and treatment of gastric cancer. Even though advanced diagnostic methods, such as X-ray barium meal examination, dual-phase helical computed tomography (CT), virtual CT, and gastroscopy, can improve gastric cancer diagnosis, their sensitivity and specificity are low for early discovery

of gastric cancer. Under such circumstances, gastric cancer can be found only after cancer cells have metastasized to their surrounding tissues, or whole-body aggravation occurs. Furthermore, cancer biological markers have a rather low positive rate for the early diagnosis of gastric cancer. At present, the sensitivity of cancer biological markers is only about 18%-40%. Combined examination of multiple cancer biological markers can achieve a sensitivity of 60%-80%. However, since the false positive rate is rather high, it is difficult to employ it as a biological index for the early diagnosis of gastric cancer[18-21]. We used the MALDI technique and WCX magnetic beads to examine gastric cancer samples, which can accurately identify gastric cancer. A difference in mass spectra was found between the normal subjects and gastric cancer patients, indicating that the present method can greatly improve the diagnosis of gastric cancer and might be used in its treatment. Twenty-three saliva samples from gastric cancer patients and 18 saliva samples from healthy volunteers were examined using MALDI-TOF-MS (AutoFlex Ⅲ, from German Bruker Company) and WCX magnetic beads. The mass peaks of four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) were significantly different in gastric cancer and normal groups. The mass peak of protein 1472.78 Da was significantly higher in the gastric cancer group than in the normal group, indicating that the protein of 1472.78 Da may play an important role in the occurrence and development of gastric cancer. Among the four peaks, only the mass peak of protein 6556.81 Da in samples from gastric cancer patients was lower than that in samples from normal subjects, suggesting that the protein expression mass spectrum diagnosis model for classification of gastric cancer and non-gastric cancer can be developed by comparative analysis of mass peaks of proteins 1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da, thus providing a new tool for predicting early gastric cancer. The positive identification rate for the mass peaks of these four proteins was significantly higher than that for the common serum cancer biological markers. Since the number of gastric cancer patients and normal controls was small, a larger sample size is needed to verify the mass peaks of the four proteins identified in this study.

In conclusion, the saliva proteome technique is an attractive prospect in the early clinical diagnosis of gastric cancer. Gastric cancer can be identified at an early stage by screening the high-risk group using MALDI technique and further studies on these proteins are needed to reveal their clinical value. Earlier detection and treatment of gastric cancer are important in decreasing the death rate. However, the saliva proteome technique needs to be further studied, because its cost is rather high and some other problems remain to be solved.

COMMENTS

Background

Gastric cancer is one of the most common cancers and threatens human health. At present, available tumor markers have a low sensitivity and a relatively high false-positive rate for the diagnosis of gastric cancer, thus cannot predict early gastric cancer. Therefore, it is necessary to find new methods to predict early stage gastric cancer.

Research frontiers

Saliva is an important and necessary body fluid. Studies on saliva proteomics have received extensive attention worldwide. At present, markers of early stage gastric cancer are a hot topic of investigation around the world. How to find effective and better methods that can be applied in clinical practice has become the target of many studies. Recent studies reveal that there are more than 1000 proteins in saliva, and saliva possesses atraumatic and convenient features, and can be used as a simple and abundant resource.

Innovations and breakthroughs

A diagnosis model was developed using weak cation exchange (WCX) magnetic beads to test saliva specimens from gastric cancer patients and healthy subjects. Significant differences in the mass to charge ratio peaks of four proteins (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) were observed between gastric cancer patients and healthy subjects.

Applications

The saliva proteomic model for distinguishing gastric and non-gastric cancer can be applied to the early diagnosis of gastric cancer.

Terminology

Saliva: An important and necessary body fluid. Blood constituents such as hormones, amino acids, electrolytes, immunoglobulin and creatinine *etc*, can enter saliva through the blood barriers of the capillary walls.

Peer review

The authors studied the differences in saliva protein spectra between normal subjects and gastric cancer patients. The study is valuable in aiding early diagnosis of gastric cancer. The method for screening patients with the risk of gastric cancer is simple, fast, and easy to use. Therefore, this study is innovative and practical, and the method developed by the authors can be used in the early diagnosis of gastric cancer.

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Combined treatment of oxaliplatin and capecitabine in patients with metastatic esophageal squamous cell cancer

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Abstract

AIM: To investigate the efficacy and side effects of the combined therapy of oxaliplatin and capecitabine in patients with metastatic esophageal squamous cell cancer (ESCC) and the survival of the patients.

METHODS: Sixty-four patients (median age of 63 years) with histological or cytological confirmation of ESCC received oxaliplatin 120 mg/m^2 intravenously on day 1 and capecitabine 1000 mg/m^2 orally twice daily on days 1 to 14 in a 21-d treatment cycle as palliative chemotherapy. Each patient received at least two cycles of treatment. The efficacy, side effects and patient survival were evaluated.

RESULTS: The partial response (PR) rate was 43.8% (28/64). Stable disease (SD) rate was 47.9% (26/64), and disease progression rate was 15.6% (10/64). The clinical benefit rate (PR + SD) was 84.4%. The main toxicities were leukopenia (50.0%), nausea and vomiting (51.6%), diarrhea (50.0%), stomatitis (39.1%), polyneuropathy (37.5%) and hand-foot syndrome (37.5%). No grade 4 event in the entire cohort was found. The median progression-free survival was 4 mo, median overall survival was 10 mo (95% CI: 8.3-11.7 mo), and the 1- and 2-year survival rates were 38.1% and 8.2%, respectively. High Karnofsky index, single metastatic lesion and response to the regimen indicated respectively good prognosis.

CONCLUSION: Oxaliplatin plus capecitabine regimen is effective and tolerable in metastatic ESCC patients. The regimen has improved the survival moderately and merits further studies.

Key words: Oxaliplatin; Capecitabine; Metastatic esophageal squamous cell cancer; Survival analysis

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INTRODUCTION

Esophageal cancer, which has the highest incidence and mortality worldwide[1-4] is one of the most common malignant tumors in China. Linzhou (formerly known as Linxian) and nearby cities, such as Anyang and Huixian in Henan Province of northern China have been well recognized as the highest incidence area for esophageal squamous cell carcinoma (ESCC) in the world; the average incidence rates for men and women are 161 and 103 per 100 000, respectively[5].

Due to the lack of obvious early symptoms, the patients were often diagnosed at advanced stages, more than half of them with metastasis[6]. The recurrence and metastasis rate after treatment of esophageal cancer have the trend to ascend in recent years. In 2007, Grunberger *et al*[7] have confirmed that palliative chemotherapy can prolong the survival of stage Ⅳ

esophageal cancer patients, relieve their symptoms and improve their quality of life. Nevertheless, no optimizing chemotherapy regimen has been developed so far, the combined regimens based on cisplatin and 5-FU has been used frequently, with an effective rate of about 25.0%-33.0%[8,9]. Squamous cell esophageal cancer is the most common histology in China, and the constituent ratio is different from that in Europe and America. Some experts state that there are complete differences between esophageal adenocarcinomas and squamous cell cancer, such as the treatment protocol and prognosis. Therefore, the focus must be laid on the study of palliative chemotherapy of metastatic ESCC.

Oxaliplatin is a kind of chemotherapeutic drug belonging to the third generation of platinum compounds, which has played an important role in the treatment of colon cancer and other solid tumors[10,11]. Oxaliplatin's side chain is substituted by the diamino-cyclohexane radical (DACH). Therefore, compared to cisplatin, DACH-platinum combines to DNA much faster with stronger cell toxicity, which has no cross tolerance with cisplatin and no oto-renal toxicity. Furthermore, it has a synergistic effect with 5-FU, with slight digestive tract reaction and hematotoxicity. Its common side effect is reversible peripheral nerve paresthesia. Oral capecitabine can be rapidly absorbed as an intact molecule in the gastrointestinal tract and most of a given dose of capecitabine is initially hydrolyzed in the liver by a carboxylesterase to 5'-deoxy-5-fluorocytidine (5'-DFCR) without bioactivity. Cytidine deaminase, an enzyme found in many tissues, including tumors, converts 5'-DFCR to 5'-DFUR. Certain human carcinomas express the enzyme thymidine phosphorylase in higher concentrations than the surrounding normal tissues, which potentially converts 5'-DFUR to higher concentrations of active 5-fluorouracil (5-FU) within these tumors.

This study aims to explore the efficacy and toxic reaction of the combined treatment of oxaliplatin and capecitabine in metastatic ESCC and the survival of the patients. The results will be used to supply information and instruction for clinical treatment.

MATERIALS AND METHODS

Patients

From January 2003 to January 2006, 64 patients (45 males and 19 females) with histological or cytological confirmation of metastatic ESCC received oxaliplatin plus capecitabine therapy. The median age of the patients was 63 years (27cases under 60 years and 37 cases over 60 years). The metastatic sites of ESCC patients were lymph node, bone, liver, lung, membrana pleuralisa, abdominal membrane, adrenal gland, skin and soft tissue. Among these patients, 42 had single-site metastasis and 22 had multi-site metastases. Karnofsky performance status (KPS) of the patients was between 60 and 100 (60-80 in 42 patients and 90-100 in 23 patients). Before the study, 28 patients had received no chemotherapy, and 36 had received previous chemotherapy, and oxaliplatin and capecitabine treatment was excluded.

All patients were required to take pathological examinations, upper gastrointestinal tract barium meal perspective, computed tomography (CT) for neck thorax and abdomen, magnetic resonance imaging or CT for skull, emission computerized tomography for bone, blood routine test, liver-renal function test, electrocardiography (ECG) and other routine tests.

Treatment

All patients received oxaliplatin and capecitabine as follows: oxaliplatin 120 mg/m^2, infused on day 1; capecitabine 1000 mg/m^2, taken orally twice a day on days 1-14. Before taking oxaliplatin, the patients received 5-hydroxy-tryptamine inhibitors to prevent vomiting. During the medication, the patients should keep their body warm, avoid cold drinks, and take vitamin B6 100 mg orally three times a day with capecitabine to prevent and decrease the occurrence of extremity syndrome. Blood routine and liver-renal function tests should also be performed, and abnormal tests should be managed to accomplish the chemotherapy. Patients with bone metastasis should receive the radiotherapy and diphosphonate simultaneously in the 21-d cycle treatment. Each patient received at least two cycles of chemotherapy.

Evaluation criteria

After completion of two cycles of chemotherapy, all patients received overall check-up. Tumor response was assessed using Response Evaluation Criteria in Solid Tumors, such as the change of the tumor size, quantity and the appearance of new lesions. Toxicity was evaluated according to the Common Toxicity Criteria for acute and subacute toxicity reactions, and confirmed again at 4 wk after treatment. Patients benefited from the treatment complete response (CR) and partial response (PR) were given one or two more cycles of chemotherapy based on their agreement and tolerance. If the disease was progressive, they should receive other chemotherapeutic protocols, and optimized supportive treatment should be administered if the patients agree and are tolerant.

Follow-up

After completion of chemotherapy, all patients were followed up every 3 mo in the first year and every 6 mo in the second year by outpatient service and telephone interview till patients' death.

Statistical analysis

Overall survival, progression-free survival, death or last follow-up results were evaluated by the Kaplan-Meier method. The life table method was used to evaluate the 1-year and 2-year survival rates. Single factor was compared by log-rank test, and multi-factor was analyzed by Cox regression proportional hazard model.

RESULTS

Short-term effects

All patients were evaluated for short-term effects and toxicity. There was no CR; 28 patients (43.8%) had

Table 1 Toxicities of oxaliplatin plus capecitabine in 64 cases of metastatic ESCC (*n* %)

Side effects	Grade				
	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Nausea and vomiting	31 (48.4)	21 (32.8)	12 (18.8)	0 (0.0)	0 (0.0)
Diarrhea	32 (50.0)	20 (31.3)	12 (18.7)	0 (0.0)	0 (0.0)
Aspherinia	44 (68.7)	11 (17.2)	9 (14.1)	0 (0.0)	0 (0.0)
Leukopoenia	32 (50.0)	20 (31.3)	10 (15.6)	2 (3.1)	0 (0.0)
Thrombocytopenia	46 (71.9)	11 (17.2)	7 (10.9)	0 (0.0)	0 (0.0)
Nerve toxicity	40 (62.5)	14 (21.9)	9 (14.1)	1 (1.5)	0 (0.0)
Hand-foot syndrome	40 (62.5)	13 (20.3)	11 (17.2)	0 (0.0)	0 (0.0)
Alopecia	60 (93.8)	4 (6.2)	0 (0.0)	0 (0.0)	0 (0.0)
Mucositis of mouth	39 (60.9)	18 (28.1)	7 (10.9)	0 (0.0)	0 (0.0)
Abnormal liver function	56 (87.6)	7 (10.9)	1 (1.5)	0 (0.0)	0 (0.0)

Table 2 Prognostic single factor analysis of ESCC

Prognostic factor	Number	Survival rate (%)		MST (mo)	*P*
		1-yr	2-yr		
Sex					0.713
Male	42	38.1	7.1	10.0	
Female	119	26.3	10.5	8.5	
Age (Yr)					0.887
< 60	26	30.8	11.5	9.0	
⩾ 60	35	42.9	5.7	10.0	
KPS					< 0.001
60-80	40	22.5	0.0	8.0	
90-100	21	66.7	23.8	13.5	
Metastasis					< 0.001
Mono-site	40	52.5	12.5	12.5	
Poly-site	21	9.5	0.0	6.5	
Prechemotherapy					0.969
Yes	27	40.7	3.7	10.0	
No	34	35.3	11.8	9.0	
Therapeutic effect					
PR	27	63.0	18.5	13.0	
SD	24	25.0	0.0	9.0	
PD	10	0.0	0.0	6.0	

PR, 26 patients (40.6%) demonstrated stable disease (SD) and 10 (15.6%) patients presented with cancer progression. The effectiveness rate was 40.6% and the overall clinical benefit rate was 84.4%.

Toxic and side effects

The main side effects of chemotherapy were alimentary tract reaction such as nausea, vomiting and diarrhea, and different grade bone marrow suppression. The occurrence of nausea and vomiting was 51.6%, and that of diarrhea was 50.0%. All side effects were slight or moderate. The main bone marrow suppression was leukopenia. The incidence rates of grade Ⅰ, Ⅱ, Ⅲ and Ⅳ leucopenia were 31.3%, 15.6%, 3.1% and 0.0%, respectively. One female patient had the symptom of digit anesthesia and anodynia late in the second cycle of chemotherapy, but this relieved gradually without treatment. The others only had grade Ⅰ or Ⅱ nerve toxicity such as dead limb, dysesthesia, and cold sensitivity. All of these recovered soon during the intermission of chemotherapy. No severe hand-foot syndrome occurred. The incidence rate of slight and moderate hand-foot syndrome was 37.5%. The other toxicities were grade Ⅰ or Ⅱ tolerable oral mucositis (39.1%), liver function abnormality (1.4%) and alopecia (6.2%). There was no renal function abnormality and death related to chemotherapy (Table 1).

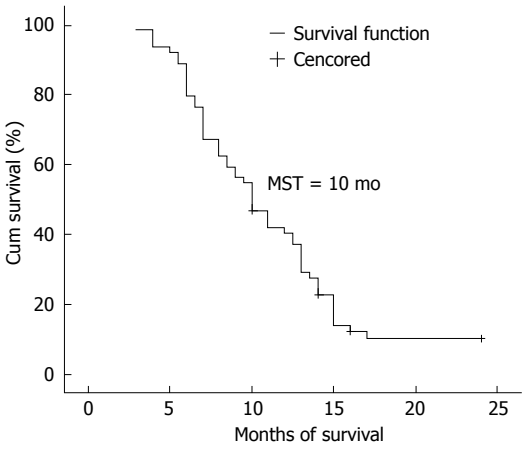

Figure 1 Cumulative survival curve of ESCC patients.

Survival analysis

The 64 patients were all followed up either for 2 years or until death. The follow-up rate was 100.0% (Figure 1). The median progression-free survival was 4.0 mo, and the median overall survival was 10.0 mo (95% CI: 8.3-11.7 mo). The 1- and 2-year survival rates were 38.1% (24/63) and 8.2% (5/61), respectively. Kaplan-Meier monofactorial analysis indicated that there was a statistical significance between the influence of KPS index, metastasis and short-term effect and survival ($P \leqslant 0.0001$), but there was no statistical significance between the influence of sex, age and therapy and survival ($P > 0.05$), (Figure 2). Cox regression proportional hazard model polyfactorial analysis indicated that KPS index, the number of tumor metastasis loci and short-term effect ($P < 0.001$) were independent survival prognostic factors, while sex, age and former therapy ($P > 0.05$) were not (Tables 2 and 3).

DISCUSSION

It is very important to treat enteric tumors by oxaliplatin plus capecitabine. There are few reports about this protocol used in esophageal cancer[12,13]. As a result of different pathological types, there have been some reports about combined treatment of oxalipatin and capecitabine for esophageal adenocarcinoma. However, there has been no report about this protocol for ESCC. Compared with other treatment of advanced ESCC, our effectiveness rate is slightly lower than that of protocol of paclitaxel and cisplatin reported by Huang *et al*[14], but higher than that of combined regimens based on cisplatin and 5-FU, as well as irinotecan and cisplatin, and similar to that of FOLFOX 4. The median overall survival is longer and the 1-year survival rate is a little higher in our study than the regimens based on cisplatin and 5-FU, as well as FOLFOX 4, both of which are frequently applied clinically. Moreover, our regimen has fewer side effects.

Table 3 Results of proportional hazards regression model

Factor	Regression coefficient	Standard error	Wald	DOF	*P*	Exp (β)	95% CI
Sex	-0.439	0.361	1.475	1	0.225	0.645	0.318-1.309
Age	-0.151	0.342	0.194	1	0.659	0.860	0.440-1.682
KPS	-1.449	0.342	17.906	1	< 0.001	0.235	0.120-0.459
Metastasis	1.932	0.390	24.497	1	< 0.001	6.902	3.212-14.833
Pre-chemotherapy	-0.235	0.291	0.653	1	0.419	0.790	0.447-1.398
Short-term effect	0.972	0.254	14.610	1	< 0.001	2.645	1.606-4.354

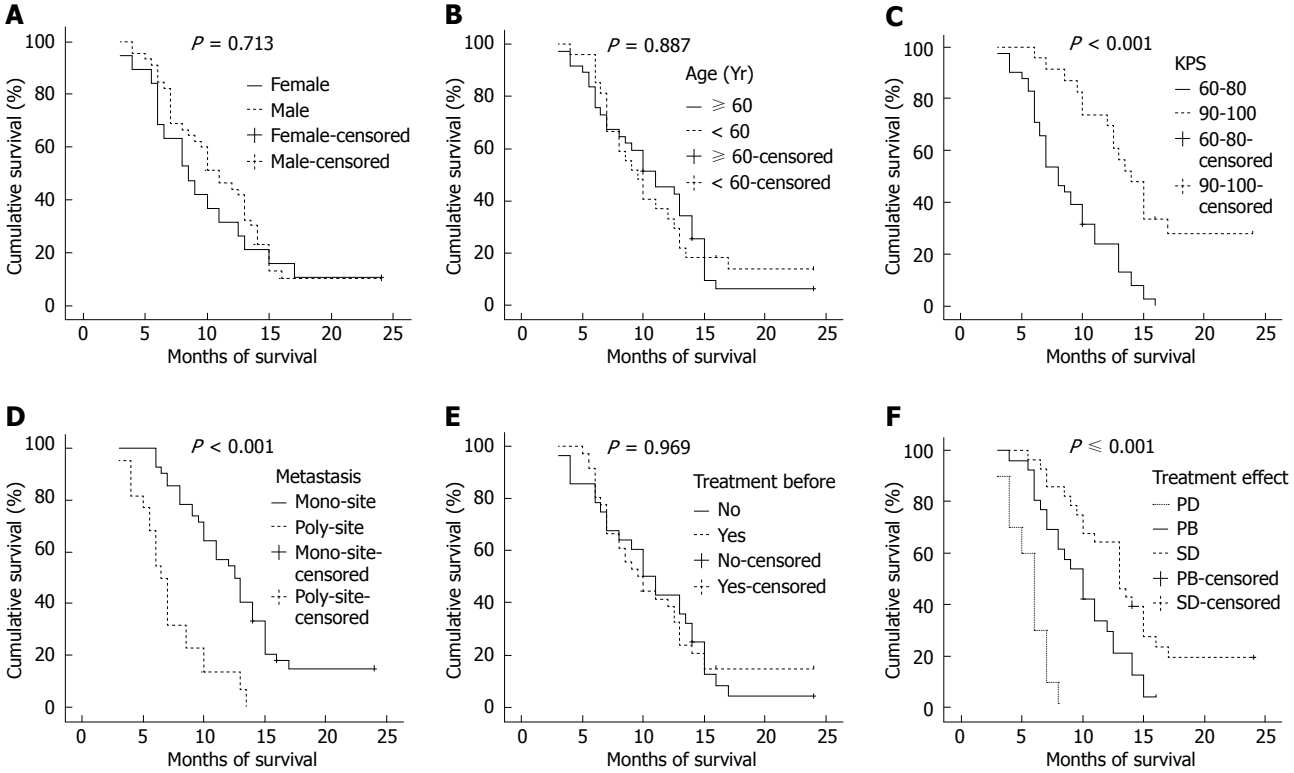

Figure 2 Cumulative survival curve. A: Cumulative survival curve of male and female ESCC patients; B: Cumulative survival curve of ESCC patients at different ages; C: Cumulative survival curve of ESCC patients with different KPS; D: Cumulative survival curve of ESCC patients with poly/mono-site metastasis; E: Cumulative survival curve of ESCC patients with or without pre-treatment; F: Cumulative survive curve of ESCC patients with different short-term effects.

Mauer *et al*[10] reported that oxaliplatin and 5-FU protocol (oxaliplatin 85 mg/m^2 iv, 5-FU 400 mg/m^2 iv quickly and then 600 mg/m^2, iv for 22 h, on day 1 and 2), has better results. The PR rate was 40.0%, the median overall survival was 7.1 mo, and 1-year survival rate was 31.0%. The main toxicities were neutrocytopenia (grade Ⅳ, 29.0%,) and peripheral neuropathy (grade Ⅱ-Ⅲ, 26.0%).

Huang *et al*[14] used paclitaxel and cisplatin regimen (paclitaxel 175 mg/m^2 , iv less than 3 h on day 1; cisplatin DDP 40 mg/m^2, iv on day 2 and 3; and repeated every 3 wk), with a PR rate of 55.5%. Of seven patients with severe neutrocytopenia, one patient died of grade Ⅳ neutrocytopenia.

Polee *et al*[15] used cisplatin, etoposide and 5-FU regimen (cisplatin DDP 80 mg/m^2, iv on day 1; etoposide 125 mg/m^2, iv on day 1 and 200 mg/m^2 , iv on day 3 and 5; 5-FU 375 mg/m^2, iv on days 1-4; folic acid 30 mg, taken orally every 4 h, on days 1-4; and the cycle was repeated every 4 wk), the PR rate was 34.0%, the median overall survival was 9.5 mo, and the 1-year survival rate was 36.0%. The main toxicities were leukopenia (grade Ⅲ-Ⅳ, 16.0%), fever related to leukopenia (19.0%), thrombocytopenia (grade Ⅲ-Ⅳ, 7.0%), mucositis (grade Ⅲ-Ⅳ, 23.0%), nausea and vomiting (grade Ⅲ, 32.0%) and diarrhea (grade Ⅲ, 6.0%).

Lorenzen *et al*[16] used capecitabine (1000 mg/m^2 taken orally twice daily on days 1-14) plus intravenous docetaxel (75 mg/m^2 on day 1). The median survival was 15.8 mo (95% CI, 7.8-23.9 mo). The intent-to-treat efficacy analysis showed an overall response rate (ORR) of 46.0%.

Lee *et al*[17] used 60 mg/m^2 of CDDP iv on day 1 and capecitabine 1250 mg/m^2 taken orally twice a day on days 1-14. The ORR was 57.8% (95% CI, 43.3-72.2). The median duration of response was 4.6 (1.0-15.6) mo, follow-up of 25.7 (10.8-42.6) mo, progression time of 4.7 mo (95% CI: 2.5-7.0 mo) and the median survival time was 11.2 mo (95% CI: 8.5-13.9 mo).

Lin *et al*[18] used the regimen, composed of paclitaxel 35 mg/m^2 1 h iv on day 1, 4, 8 and 11; cisplatin 20 mg/m^2, 2 h iv on day 2, 5, 9 and 12; and 5-FU 2000 mg/m^2, leucovorin 300 mg/m^2 24 h iv on day 5 and 12; and repeated every 21 d. The median progression-free and

overall survival rates were 6.3 and 8.9 mo, respectively.

Evans *et al*[19] used docetaxel and oxaliplatin on day 1 and 8 and capecitabine individually, twice daily, on day 1-10, with each cycle repeated every 21 d. The docetaxel dose ranged from 30 to 35 mg/m^2, the oxaliplatin from 40 to 50 mg/m^2, and the capecitabine from 750 to 850 mg/m^2 twice daily. Grade 3/4 dose-limiting toxicities of diarrhea, nausea, fatigue and febrile neutropenia occurred in three of four patients at dose level 3. An intermediate dose was added (2A) and the capecitabine dose reduced to 750 mg/m^2. One of 6 patients had a dose-limiting toxicity at level 2A.

Tsai *et al*[20] used carboplatin (area under the ROC curve AUC = 2) on day 1 and 8, docetaxel (35-40 mg/m^2) on day 1 and 8, and capecitabine (500-2000 mg/m^2) on days 1-10. The maximum tolerated dose of docetaxel was 40 mg/m^2 on day 1 and 8; carboplatin, AUC = 2 on day 1 and 8; and capecitabine, and 1500-2000 mg/m^2 on days 1-10 in a 21-day cycle. Ten of 25 patients who could be evaluated (40.0%) responded and eight of 14 patients treated at the final dose level responded (57.0%).

Lee *et al*[21] used two cycles of XP induction chemotherapy, consisting of capecitabine 1000 mg/m^2 twice daily on days 1-14, and cisplatin 60 mg/m^2 iv on day 1, every 3 wk. Patients classified as M1a and M1b (non-visceral lymph node metastases) were treated with 54 Gy radiotherapy, concurrently with weekly capecitabine 800 mg/m^2 twice daily on days 1-5 and cisplatin 30 mg/m^2 iv on day 1 during radiation. Patients classified as M1b (visceral metastases) were treated with chemotherapy only until disease progression or intolerance to chemotherapy. The median time of progression was 7.8 mo (95% CI, 6.0-9.5 mo) and the median overall survival was 12.0 mo (95% CI, 9.0-15.0 mo).

Evans *et al*[22] used a regimen comprised of docetaxel 40 mg/m^2, on day 1 and 8, carboplatin (AUC = 2) on day 1 and 8, and capecitabine 2000 mg/m^2, on days 1-10 in a 21-day cycle. The median survival was 8.0 mo (95% CI, 5.5-13.0 mo), and the 1-year survival rate was 36.0%.

In our study (oxaliplatin 120 mg/m^2, iv on day 1; capecitabine 1000 mg/m^2, taken orally twice a day on days 1-14; and repeated every 3 wk), the rate was 43.8%, the median overall survival was 10 mo, and the 1-year survival rate was 38.1%. The main toxicities were leukopenia (grade Ⅲ, 31.0%) and neuro-toxicity (grade Ⅲ, 1.5%).

Capecitabine can be taken orally, so the protocol has superiority in medication. The mono-factorial analysis by Kaplan-Meier indicates that patients with low KPS and multi-locus metastases benefit little from this therapy. The short-term effect indicates that the prognosis demonstrates the importance of prompt, objective and precise therapeutic effect in clinical practice. Cox regression proportional hazard model poly-factorial analysis indicates that KPS index, the number of tumor metastasis locus and short-term effect are independent survival prognostic factors.

Our results demonstrate that oxaliplatin plus capecitable regimen has the advantage of good short-term effects, convenient administration and minor side effects in metastatic ESCC. The functional status of prior treatment, the number of tumor metastasis loci and short-term effects are independent survival prognostic factors.

COMMENTS

Background

Esophageal cancer which has the highest incidence and mortality worldwide is one of the most common malignant tumors in China. Esophageal squamous cell cancer (ESCC) is the most common histology. It has been confirmed that palliative chemotherapy can prolong the survival of stage Ⅳ esophageal cancer patients, relieve their symptoms and improve their quality of life. Nevertheless, no optimizing chemotherapy regimen has been available so far, the combined regimens based on cisplatin and 5-fluorouracil have been used frequently, but the effectiveness rate is only about 25.0%-33.0%.

Research frontiers

Oxaliplatin is a kind of chemotherapeutic drug belonging to the third generation of platinum compounds, which has played an important role in the treatment of colon and rectum cancer and other solid tumors. It has a synergistic effect with lesser digestive tract reaction and hematotoxicity. Capecitabine, which has milder side effects, can be taken orally and is rapidly absorbed as an intact molecule in the gastrointestinal tract. Therefore, the combined regimen of oxaliplatin and capecitabine may produce more clinical benefits.

Innovations and breakthroughs

This study explored the efficacy and toxic reaction of oxaliplatin plus capecitabine in the treatment of patients with metastatic ESCC and the survival of the patients. The partial response (PR) rate was 43.8% (28/ 64). Stable disease (SD) rate was 47.9% (26/64), and disease progression rate was 15.6% (10/64). The clinical benefit rate (PR + SD) was 84.4%. No grade Ⅳ side effect in the entire cohort was found. The results can be used to supply information and instruction for clinical treatment.

Applications

Higher response and survival rate, and lower rate of toxicity were obtained by the combined treatment in this study. Capecitabine can be taken orally, therefore, that this treatment can be used clinically.

Terminology

RECIST stands for Response Evaluation Criteria in Solid Tumors. KPS stands for Karnofsky performance score.

Peer review

This is the first report to examine the efficacy and toxicity of the combined therapy of oxaliplatin and capecitabin in patients with metastatic ESCC. Higher response and survival rate, and lower rate of toxicity were obtained by this treatment than the other treatment protocols reported previously. It seems that this treatment has become a candidate for phase Ⅲ study. Furthermore, since this treatment can be given on an outpatient basis, this study has great value.

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CASE REPORT

An obstructing mass in a young ulcerative colitis patient

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Abstract

We present a case of a 19-year-old female who developed subacute obstruction due to giant inflammatory polyps, having undergone treatment for left-sided ulcerative colitis. This is followed by a review of the literature.

Key words: Pseudopolyp; Ulcerative colitis; Inflammatory polyps; Giant intestinal polyposis

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INTRODUCTION

Giant inflammatory polyps occur rarely in ulcerative colitis patients. The presentation is often insidious and the endoscopic appearance can be alarming. The following case illustrates these points.

CASE REPORT

A 19-year-old female student presented to Hemel Hempsted General Hospital in May 2006 following a 20-d history of bloody diarrhea. The patient was on no regular medications at the time of admission. She had no significant past medical history apart from iron deficiency anemia [hemoglobin 7.4 g/dL, mean corpuscular volume (MCV) 67.3] with raised inflammatory markers (C-reactive protein; CRP, 89 mg/L). Flexible sigmoidoscopy demonstrated left-sided colitis and subsequent biopsies confirmed it to be ulcerative colitis. She received a short course of oral prednisolone (40 mg) and balsalazide (2.25 mg *tds*) with rapid improvement. At the time of her follow-up appointment in July 2006, she was asymptomatic.

At a subsequent clinical appointment in September 2006, the patient complained of increasing nausea, lethargy and borborygmi. Blood taken at the time revealed a microcytic anemia (hemoglobin 9.8 g/dL, MCV 78) despite being on ferrous sulfate and an albumin of 19 g/L. Her CRP was < 3 mg/L. She was readmitted to our hospital in November 2006 having developed 10 episodes of watery diarrhea a day. She was treated with steroids for exacerbation of colitis, started on azathioprine and discharged from our hospital. Over the subsequent months, the patient continued to complain of worsening lower abdominal pain, vomiting and lethargy. She was readmitted for investigation and her admission blood analysis revealed CRP < 3 mg/L. Her abdominal X-ray was unremarkable. She underwent a flexible sigmoidoscopy to assess the extent of inflammation. There was no evidence of active ulcerative colitis, but the discovery of a mass at the splenic flexure prompted further imaging with computed tomography (Figure 1). Because of the obstructive nature of the mass and the fact that the mass extended along the transverse colon, the patient underwent an extended right hemicolectomy with primary anastomosis (Figure 2). The histology of the mass revealed it to be an inflammatory pseudo-polyp with no evidence of dysplasia. She subsequently made a good postoperative recovery with a normalization of all blood results.

DISCUSSION

Giant inflammatory polyps (GIPs), also known as filiform polyposis or pseudo-polyps, are defined as being more than 1.5 cm in diameter. First described in 1965[1,2], they can occur in both ulcerative colitis and Crohn's disease,

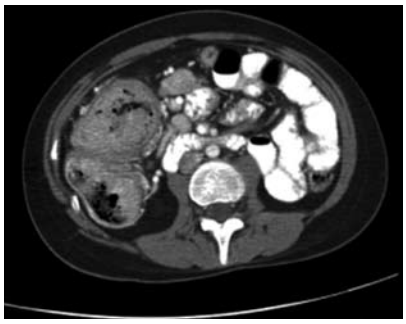

Figure 1 Abdominal computed tomography showing a mass at the splenic flexure.

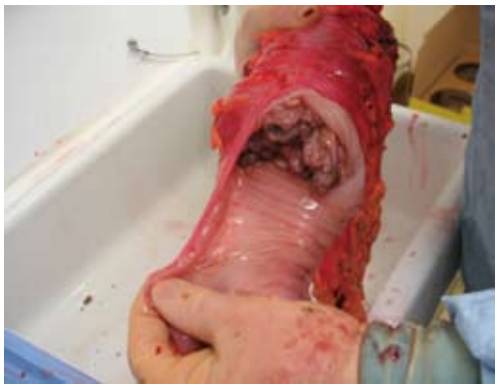

Figure 2 Resected specimen revealing an inflammatory pseudo-polyp with no evidence of dysplasia.

although the former is more common. They occur most commonly in females with pancolitis at the age of 20-40 years, diagnosed 1-5 years prior to presentation with GIPs. There is a predilection for the transverse colon, although the condition has been described at all colonic sites. GIPs can present in a number of different ways, including crampy abdominal pain, anemia, obstruction, hypoproteinemia and palpable abdominal mass[3-8].

Its presentation and endoscopic findings may mimic those of a colonic tumor. There are no pathognomonic signs to confidently differentiate colonic pseudo-polyp clinically, radiologically or endoscopically from villous adenoma, dysplasia-associated lesion or mass or carcinoma[9]. The pathogenesis is deemed to be abnormal healing in the form of enthusiastic post-inflammatory regeneration[9-11]. GIPs have been found in both quiescent and active diseases[12] which may represent detection at different stages in their development. Balazs has found further evidence for this[13] from the histopathological analysis of GIPs which shows changes similar to those described in delayed type hypersensitivity[14]. Treatment is currently surgical in all previously described case reports, as most of the patients present with obstruction, and because of the size of the polyp.

We believe that GIPs should be suspected more often in young patients with colitis presenting with obstruction. Hypoalbuminemia is an interesting aspect that has been previously reported and attributed to an etiology similar to that of Menetrier's disease. Although non-specific in the appropriate clinical context, its presence should add further suspicion for the presence of GIPs[15,16].

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CASE REPORT

Extensive hepatic-portal and mesenteric venous gas due to sigmoid diverticulitis

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Abstract

Hepatic portal venous gas is most often associated with extensive bowel necrosis due to mesenteric infarction. Mortality exceeds 75% with this condition. The most common precipitating factors include ischemia, intra-abdominal abscesses and inflammatory bowel disease. In this report, we present a 75-year-old woman with extensive hepatic portal and mesenteric venous gas due to colonic diverticulitis. She had a 10-year history of type Ⅱ diabetes mellitus and hypertension. She was treated by sigmoid resection and Hartmann's procedure and discharged from the hospital without any complications.

Key words: Hepatic portal vein; Gas; Sigmoid diverticulitis; Computed tomography

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INTRODUCTION

Hepatic portal venous gas (HPVG) is a rare condition and traditionally regarded to be an ominous finding of impending death, with highest mortality reported in patients with underlying bowel with ischemia[1-6]. Colonic diverticulitis is an inflammatory condition and in very rare cases can be associated HPVG[6-9]. HPVG can be due to two mechanisms: gas under pressure in the bowel lumen or to an alteration of the mocosa, allowing the gas to enter the portal system through the mesenteric veins; or gas-forming bacteria in intra-abdominal abscesses with or without a related pylephlebitis[3-6]. If there is an underlying intramesocolic abscess and perforation in complicated diverticulitis, the prognosis is favorable, but the prognosis of HPVG due to septic thrombophlebitis and gas-forming organisms is poor[6]. Another factor affecting HPVG and its prognosis is the existence of a long-term chronic disease, such as chronic renal failure, diabetes mellitus and hypertension[5]. It has been reported that long-term chronic diseases decrease immune functions and alter the intestinal microbial flora and tolerance capability of the HPVG patients, which might lead to fatality[5,10].

We report the case of a 75-year-old woman with type Ⅱ diabetes mellitus and hypertension presenting with extensive hepatic, portal and mesenteric venous gas due to sigmoid diverticulitis.

CASE REPORT

A 75-year-old woman was seen in the emergency department with a 4-d history of mild abdominal pain and fever. Except for her temperature (38.2℃), her vital signs were normal. She had a 10-year history of hypertension and type Ⅱ diabetes mellitus, and antihypertensive drugs and insulin therapy had kept her blood pressure and blood sugar level within normal ranges. On physical examination, she had mild tenderness in the left lower quadrant but no localizing peritoneal signs. Her serum C-reactive protein level was 220 mg/L, her platelet count was 55 000/mm^3, and other laboratory findings were normal. A computed tomography (CT) scan of the abdomen revealed multiple gas foci in the main hepatic-portal vein, portal vein branches (Figure 1) and superior mesenteric (Figure 2A), splenic (Figure 2B) and inferior

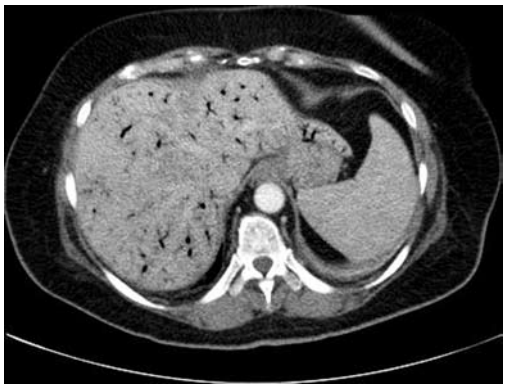

Figure 1 Extensive gas accumulation in the hepatic-portal vein branches.

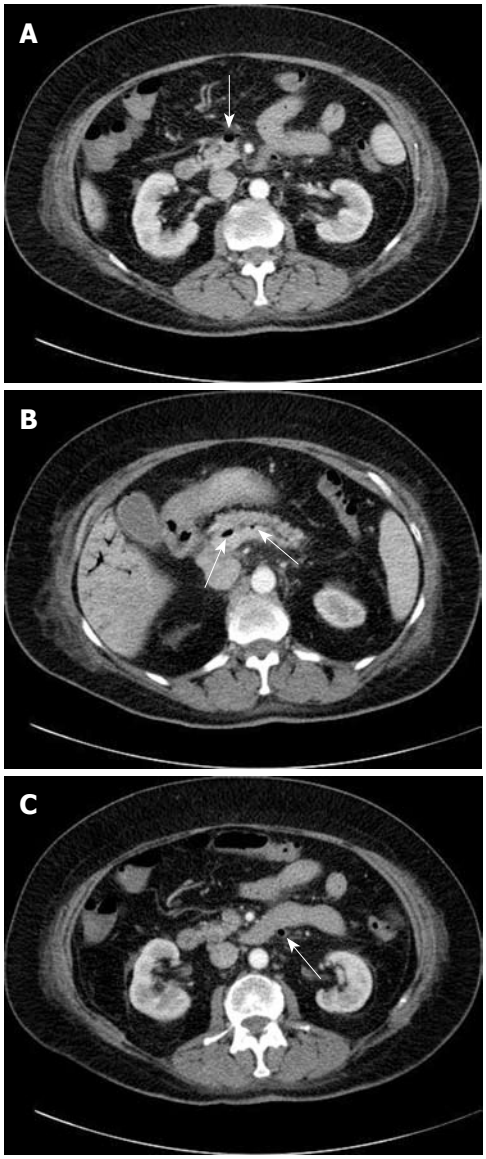

Figure 2 Gas observed. A: Superior mesenteric vein (arrow); B: Splenic vein (arrows); C: Inferior mesenteric vein (arrow).

mesenteric veins (Figure 2C). Sigmoid diverticulosis involved the mid and distal portions of the sigmoid colon. Slight wall thickening and inflammation suggesting localized sigmoid diverticulitis were observed (Figure 3).

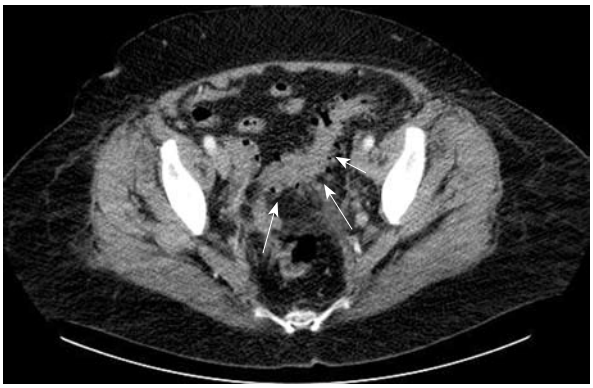

Figure 3 Diverticulosis in the sigmoid colon and mild inflammatory changes are present in the sigmoid mesocolon (arrows).

The patient was transferred to the general surgery department, started on intravenous fluid therapy and antibiotics (ceftriaxone 2 g/d and ornidazol 1 g/d), and a laparotomy was planned for the same day. The emergency laparotomy revealed mild findings of diverticulitis in the sigmoid colon. A Hartmann's procedure and end colostomy were performed. The patient was discharged on the seventh postoperative day without further complications. Her colostomy was closed 8 wk after the first operation, and she reported no problems upon examination after 3 mo.

DISCUSSION

HPVG gas was first described by Wolfe and Evans[11] in 1955 in fatal necrotizing enterocolitis in infants. This entity is most commonly associated with ischemic bowel disease. In the series of 64 patients reviewed by Liebman *et al*[1], HPVG was mainly associated with necrotizing bowel disease (72%) and found to have a 75% mortality rate. HPVG venous gas has also been associated with such entities as bowel distention, perforated ulcer, acute hemorrhagic pancreatitis, corrosive ingestion and inflammatory bowel disease such as Crohn's disease[6,12-15].

HPVG and thrombophlebitis is a rare complication of diverticulitis. Zielke *et al*[3] reported that this entity was confined to nine patients with mesocolic abscesses. In 2007, Sellner *et al*[6] reported a second review of 21 patients with complicated diverticulitis because of HPVG. In this report, 55% of cases had mesocolic abscesses and in the remaining 45%, mesocolic abscesses were absent and the patients presented with septic pylephlebitis. The authors suggested that patients with mesocolic abscess have better prognosis than patients with septic pylephlebitis. Despite our patient's symptoms, the clinical findings of diverticulitis were very slight. The CT findings revealed extensive multiple gas foci in the main portal vein, portal vein branches and superior mesenteric vein, and also revealed that the diverticulitis was milder than expected. We thought that this discordance might be due to virulence of pathogens or deficiency of the patient's immune system as a consequence of diabetes mellitus. Therefore, we decided

to perform a laparotomy on the same day. Operational findings of diverticulitis were quite limited and slight and we did not observe a mesocolic abscess. Nobili *et al*[7] suggested that if medical conservative therapy could be resolutive and the clinical status improves, the surgery could be delayed in these patients. Even though the clinical status of our patient was stable, we preferred to perform an urgent laparotomy because we thought that prognosis might be worse due to diabetes mellitus and gas-forming organisms. Chan *et al*[5] have reported that long-term chronic diseases change the microbial flora in the intestine and decrease the immune function and tolerance capability of HPVG patients, which might lead to fatality. Our patient had type Ⅱ diabetes mellitus and hypertension for 10 years, and extensive HPVG might be explained by increased aerobic and anaerobic microorganisms in the intestinal flora due to diabetes mellitus.

In conclusion, the clinical and radiological findings of HPVG associated with diverticulitis may be variable. We thought that, although the clinical status of our patient was stable, extensive HPVG in diverticulitis could be dangerous, due to a compromised immune system and alteration in intestinal flora, especially among elderly patients with chronic systemic diseases and no obvious mesocolic abscess or perforation, such as in our patient.

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CASE REPORT

A rare case of duodenal duplication treated surgically

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Abstract

Duodenal duplication, a rare congenital malformation, can also be observed in adulthood. Although it can be cystic or tubular, communicating or non-communicating, cystic and non-communicating forms are the most common. Several complications, such as obstruction, bleeding, perforation and pancreatitis, may result. Optimal treatment is total excision, although endoscopic procedures have also been described in appropriate cases. If total excision is not possible, subtotal excision and internal derivation can be performed. The 38-year-old woman presented here had occasional attacks of abdominal pain and obstruction, and we considered the diagnosis of duodenal duplication by abdominal computerized tomography. As we confirmed the diagnosis with operative findings and histopathological signs, we treated her with subtotal excision and intraduodenal cystoduodenostomy.

Key words: Duodenum; Duplication; Subtotal excision; Intraduodenal cystoduodenostomy

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INTRODUCTION

Duplications of the gastrointestinal system are rare congenital malformations observed in one out of 25 000 deliveries. About 33% of the cases are reported in adults above 20 years of age. Duodenal duplication constitutes 5%-7% of all gastrointestinal duplications. Its etiology is as yet unknown. Treatment is mainly surgical and total excision, if possible, is the procedure of choice. However, in some cases, alternative procedures, such as subtotal removal or digestive derivation, are required because of extensive size or location[1]. Here, we present a rare case of duodenal duplication in which the treatment was subtotal excision with intraduodenal cystoduodenostomy.

CASE REPORT

A 38-year-old woman who had occasional abdominal pain was referred to us with a clinical diagnosis of gastric outlet obstruction. The epigastrium was mildly sensitive on physical examination. Laboratory findings were normal but abdominal ultrasonography (US) showed gastric distension. Initial endoscopy was useless because of remnants of food. After nasogastric decompression, we repeated endoscopy but found no abnormalities. Upon abdominal computerized tomography (CT), a cystic lesion of 5 cm × 8 cm × 9 cm in diameter was observed, which extended along the lateral wall of the first and second parts of the duodenum. Remnants of food and orally taken contrast media were found within the lesion, and we observed the nasogastric tube entering the lesion through a defect between the duodenum and the cyst (Figure 1). We operated on the patient and found a cystic dilatation, 10 cm × 12 cm in diameter, anterolateral to the first and second parts of the duodenum (Figure 2A). We performed cystotomy and observed it make contact with the normally located duodenum at the posteromedial side of the cyst, through a defect of 2 cm × 2 cm in diameter. The wall between the duodenum and the cyst beneath the opening was covered with mucosa on both sides (Figure 2B). Thus, the diagnosis was cystic and communicating duodenal duplication. The wall between

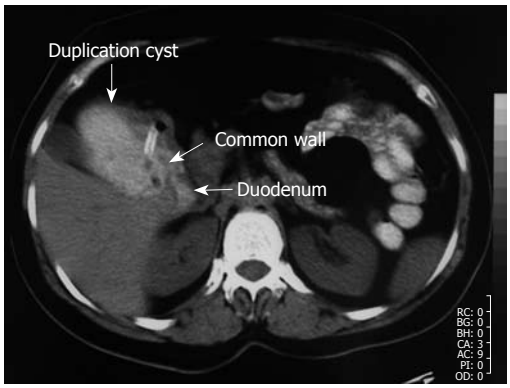

Figure 1 Oral and intravenous contrast-enhanced abdominal CT imaging. The nasogastric tube was extended into the cyst, which was filled with oral contrast medium, through the defect between the duodenum and the cyst.

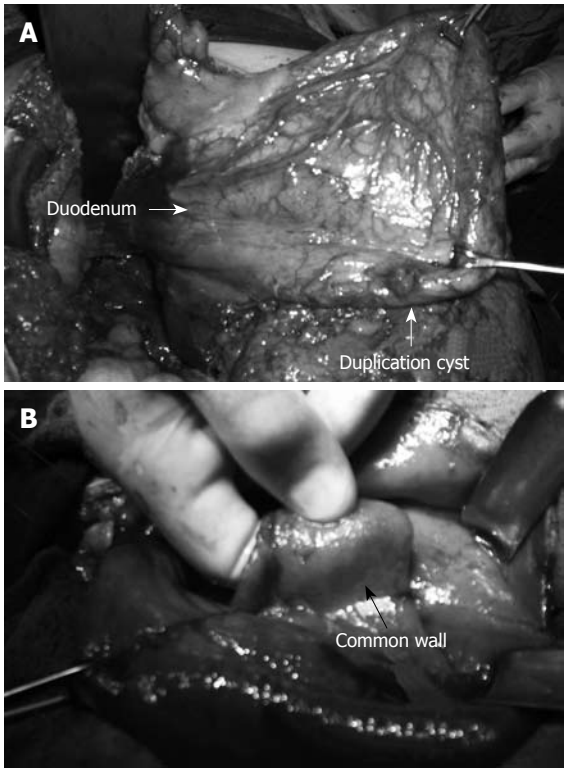

Figure 2 Operative view of cyst. A: Duodenal duplication cyst; B: Inner surface of the cyst.

the cyst and duodenum was excised and the corners were sutured to form a cystoduodenostomy. The cystic wall is excised next to the pylorus above, duodenum laterally, pancreas medially and the third part of duodenum below, while the remnant is sutured primarily. The diagnosis was confirmed histopathologically by identifying a separate mucosa with its own muscularis mucosa on both sides of the wall between the cyst and duodenum and intervening connective tissue fibers (Figure 3). The patient was without complaint after 9 mo follow-up.

DISCUSSION

Duplications of the gastrointestinal system can be observed anywhere along the alimentary tract, and they

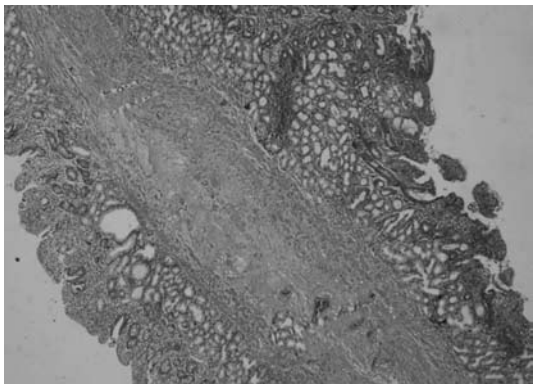

Figure 3 Common wall containing double mucosa with muscularis mucosa on each side and intervening connective tissue fibers (Hematoxylin and eosin, x 10).

are located most often in the ileum and least often in the duodenum. Duodenal duplications can be cystic or tubular, communicating or non-communicating, but the most common type is cystic and non-communicating. These are generally located at the medial border of the first and second parts of the duodenum and extend to the anterior or posterior side[1-3]. Duodenal duplication observed in our case was cystic and located in the first and second parts of the duodenum, but it was of the communicating type and located on the antimesenteric side.

A variety of clinical manifestations have been reported that are determined by the type, site and size of the duplication. Generally, patients present with a palpable mass in the abdomen, signs of intestinal obstruction, or abdominal pain. Bleeding or perforation caused by peptic ulcer and jaundice, and pancreatitis caused by biliary obstruction may also be the manifestations[1-6]. In the current case, occasional attacks of abdominal pain and gastrointestinal obstruction were present. Obstruction in non-communicating cystic duplications is defined by compression of the duplication cyst, which is distended by intracystic secretions[1]. However, in our case, which was of the communicating type, it can be described by compression of the cyst, which was filled with the gastric contents but not drained.

Although radiological methods are helpful for diagnosis, preoperative diagnosis of duodenal duplication is rarely made accurately. In barium studies, in non-communicating cysts, the first and the second parts of the duodenum can be seen as compressed and displaced by a mass, whereas, in the communicating type, the cyst itself can be observed as being filled with barium[2,7]. In the current case, if barium studies had been performed, the communication between the duplication and duodenum would have been demonstrated better. Duodenal duplication is differentiated from other cystic lesions by the "gut signature" of its wall observed by abdominal or endoscopic US. Gut signature refers to the layered pattern of the wall, with the hyperechoic inner layer representing the submucosa and the hypoechoic outer layer representing the smooth muscle[8-10]. Peristalsis

of the cyst wall noted upon real-time US is strongly suggestive of a duplication cyst[11]. US is an operator-dependent method and unfortunately it was not helpful in the diagnosis of our case. CT is valuable in identifying the type, location and the size of the duplication cyst. In the differential diagnosis of duodenal duplication, one should be mindful of choledochocele, pancreatic pseudocyst and intraluminal diverticulum[12]. In our case, the location of the cystic lesion and CT images made us think of cystic, communicating duodenal duplication. Although magnetic resonance imaging (MRI) and gastroduodenoscopy are the other modalities that can be used for diagnosis, CT images were sufficient and MRI was not required in our case. Our not having a diagnostic sign upon endoscopy might have been caused by the fact that the endoscope we used was without lateral vision.

In spite of the diagnostic workup performed before the operation, accurate diagnosis of duodenal duplication is by histological examination. According to the analysis made by Merrot *et al*[1], two types of intra- or juxta-duodenal duplications occur: (1) a common wall formed by two separate mucosae with their own muscularis mucosa and a layer of intervening connective tissue; and (2) a common wall that comprises two mucosal layers with two smooth muscle layers, but that also contains biliary and pancreatic ducts. In our case, histological diagnosis of intraduodenal duplication was made by observation of the mucosa on both sides, each with its own muscularis mucosa, and connective tissue fibers between the two layers.

Management of duodenal duplications depends on the volume, type, location and proximity to the duodenal wall, pancreas or biliary ducts. If there is no communication between the mass and the biliary or pancreatic ducts, and if the vasculature allows, total resection is the procedure of choice. However, if it is not possible, partial resection or internal derivation must be carried out. In partial resection, all of the cyst wall is removed wherever possible, while the area of maximum adherence to the duodenum is preserved[1,13-15]. In our case, duplication was communicating and preservation of duodenal continuity would have been impossible if total resection had been performed. Thus, we performed partial resection with maximal removal of the cystic wall, which allowed secure closure. As the diameter of the communication was not efficient for adequate drainage of the cyst, the common wall between the cyst and the duodenum was excised and the corners were sutured to form a large cystoduodenostomy. This procedure is known as intraduodenally performed internal derivation, and it forms the basis of the endoscopic treatment of duodenal duplication[16].

In conclusion, duodenal duplication should be considered in the differential diagnosis of a patient who presents with abdominal symptoms when cystic structures neighboring the duodenum are demonstrated by radiology. Ideal treatment is total excision but, if not possible, subtotal excision and/or internal derivation should be performed.

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CASE REPORT

Deep venous thrombosis after gastrectomy for gastric carcinoma: A case report

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Abstract

The treatment of gastric carcinoma consists of neoadjuvant chemoradiation, partial gastrectomy, subtotal gastrectomy, total gastrectomy, extended resection, and postoperative chemotherapy. Currently, gastrectomy and extended lymphadenectomy is the optimal choice for late gastric carcinoma. Postoperative complications are common after total gastrectomy including hemorrhage, anastomotic leakage, fistula, and obstruction. However, deep venous thrombosis (DVT) is an uncommon complication after gastrectomy for gastric carcinoma. We describe a case of a 68-year-old female patient with DVT after gastrectomy for gastric carcinoma. The patient was treated with anticoagulants and thrombolytics and subjected to necessary laboratory monitoring. The patient recovered well after treatment and was symptom-free during a 3-mo follow-up. We conclude that correct diagnosis and treatment of DVT are crucial.

Key words: Gastric carcinoma; Gastrectomy; Deep venous thrombosis; Postoperative complication; Anticoagulant; Thrombolytic therapy; Low molecular weight heparins; Streptokinase; Warfarin sodium

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INTRODUCTION

Deep venous thrombosis (DVT) is an uncommon complication after gastrectomy for gastric carcinoma. DVT may indicate a worse prognosis. Hence, the correct diagnosis and effective methods to prevent and treat DVT are important and can reduce morbidity and mortality of the disease. Low-molecular-weight heparins (LMWHs) play a major role in the management of DVT. We report, in this paper, a case of a 68-year-old female patient with DVT after total gastrectomy for gastric carcinoma, who underwent thrombolytic and anticoagulant therapies successfully.

CASE REPORT

A 68-year-old female patient, complaining of left leg pain and tumefaction, was admitted to our surgical department in May 2007. She underwent total gastrectomy for primary gastric adenocarcinoma 3 mo ago. Physical examination revealed left leg tumefaction and pressure pain below the inguinal triangle. Type B ultrasound (Figure 1A and B) showed left leg DVT. After diagnosis, thrombolytic therapy and anticoagulant therapy were performed, in which streptokinase (600 000 U/d, days 1-3) was infused iv, LMWH (0.4 mL/d, days 1-14) was subcutaneously injected and warfarin sodium (2.5 mg/d, days 1-7) was administered orally. Laboratory monitoring showed both prothrombin time and thrombin time were normal during the thrombosis treatment The patient recovered well after the treatment. Two weeks after treatment, B type ultrasound (Figure 2) showed the partial recirculation of the left leg DVT. The patient was free of symptoms and signs of recurrent DVT during a 3-mo follow-up.

DISCUSSION

The treatment of gastric carcinoma consists of neoadjuvant chemoradiation, partial gastrectomy,

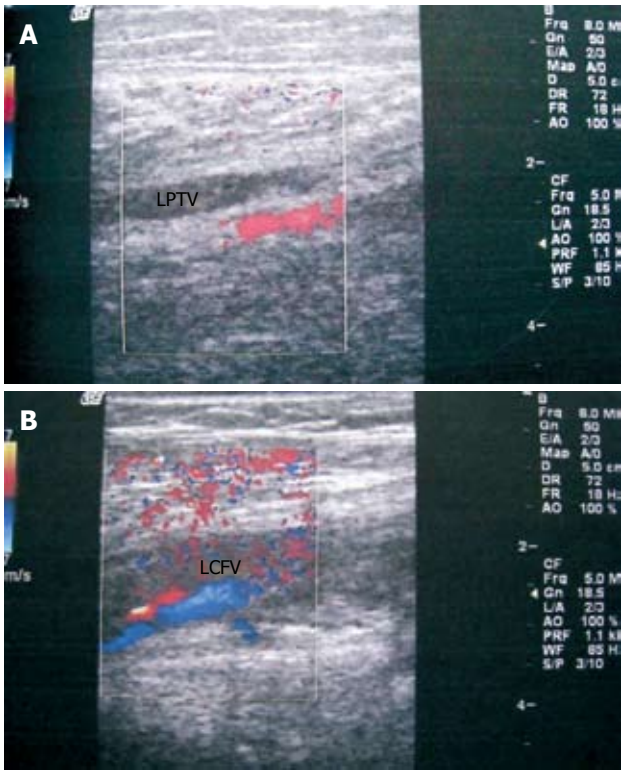

Figure 1 B type ultrasound (A and B) showed the left leg DVT.

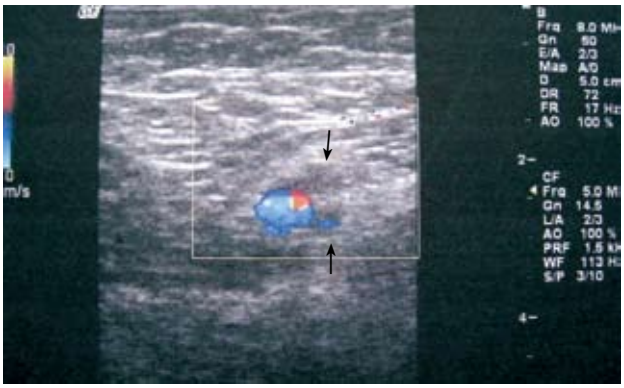

Figure 2 B type ultrasound after treatment showed partial recirculation of the left leg DVT.

subtotal gastrectomy, total gastrectomy, extended resection, and postoperative chemotherapy. Currently, gastrectomy and extended lymphadenectomy is the optimal choice for late gastric carcinoma[1]. Postoperative complications after total gastrectomy included hemorrhage, anastomotic leakage, fistula, and obstruction[2-6]. However, DVT is an uncommon complication after gastrectomy for gastric carcinoma. Venous thromboembolism (VTE), manifested as either DVT or pulmonary embolism, is an extremely common medical problem, occurring either in isolation or as a complication of other diseases or procedures[7,8]. López and Conde discussed the mechanisms and proposed that venous thrombi may be initiated on the vessel wall in the absence of anatomically overt vessel wall injury. Elevations in the levels of TF-bearing microvessels associated with inflammatory conditions would help explain the increased risk of thrombosis associated with infections and inflammatory states such as inflammatory bowel disease. The study provides an algorithm for using risk assessment as a means of determining the length and type of therapy to be used to minimize the recurrence, while diminishing the risk of simultaneous bleeding associated with anticoagulation[7]. Patients with cancer make up approximately 20% of those presenting with first-time VTE, and the presence of VTE anticipates a much poorer prognosis for patients with cancer, probably because of the morbidity associated with VTE itself and because VTE may herald a more aggressive cancer[7,9]. Chemotherapy can increase the risk of venous thrombosis in breast cancer patients. This risk increase appears to be greatest in postmenopausal patients[10]. A hypercoagulative state is observed in cancer patients, as shown by abnormal "routine" blood tests found in up to 90% of these patients, as well as increased levels of specific markers of coagulative activation[9,10].

LMWH is the drug of choice for the prevention and treatment of VTE in patients with cancer[11]. For prophylaxis in the surgical setting, a single dose of subcutaneous LMWH is as effective and safe as multiple doses of unfractionated heparin. Extending prophylaxis with LMWH beyond hospitalization was recently found to reduce the risk of postoperative thrombosis after abdominal surgery for cancer. The potential anti-neoplastic effects of LMWHs make these agents an attractive option for patients with cancer[11]. The study of López indicates that LMWH improves the survival of patients with advanced cancer through mechanisms beyond their effect as anticoagulants. As a result of their improved efficacy and safety and potential anti-neoplastic effect, LMWHs have become the anticoagulants of choice for treating VTE associated with cancer[7-9].

DVT is a severe problem in patients with cancer that complicates the management and predicts a worse prognosis. The pathophysiology of this thrombophilic state is complex due to interactions of tumor cells and their products with host cells. Risk of thrombotic complication can be reduced and survival improved by administration of anti-coagulants[8-10,12]. LMWH has simplified and improved the management of VTE, and recent studies suggest that it may improve the survival of cancer patients. This review provides an update on the primary prevention and treatment of VTE, as well as prophylaxis of central venous catheters in patients with malignancies[9,10].

DVT is a serious complication of gastrectomy and is historically associated with a high mortality. We conclude that correct diagnosis and treatment of DVT after surgery or chemotherapy are crucial.

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CASE REPORT

Signet-ring cell carcinoma of ampulla of Vater: Contrast-enhanced ultrasound findings

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Abstract

Signet-ring cell carcinoma (SRCC) of ampulla of Vater is extremely uncommon, and less than 15 cases have been reported so far in literature. It mainly occurs in elderly people (median age 57 years). We report a rare case of SRCC of the ampulla of Vater in a 38-year-old woman who presented with a small tumor at the Vater, discovered by the contrast-enhanced ultrasound (CEUS). Histopathological examination showed prominent signet-ring features. We also describe the imaging features of SRCC of ampulla of Vater in CEUS.

Key words: Signet-ring cell carcinoma; Ampulla of Vater; Contrast-enhanced ultrasound

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INTRODUCTION

Signet-ring cell carcinoma (SRCC) usually occurs in the gastrointestinal tract. The World Health Organization (WHO) defines it as a special type or a variant of gastrointestinal adenocarcinoma. SRCs may exist alone or coexist with any other types of malignant gastrointestinal tumors. SRCC is very rarely found among carcinomas of the ampulla of Vater. Here, we describe one patient with SRCC in the ampulla of Vater, which was found by contrast-enhanced ultrasound (CEUS). This is the first case reported in literature, which was successfully diagnosed with CEUS.

CASE REPORT

A 38-year-old woman was hospitalized because of pruritus for 13 d, and dermatic and scleral jaundice with urine the color of bean oil for 5 d. The stool s had a silver color. The patient had nausea but without vomiting, fever and abdominal pain. She lost weight of about 3 kg in 1 mo. She had a history of surgery for left breast adenoma at another institution several years ago.

Physical examination revealed mucocutaneous jaundice without tenderness in the epigastrium. The laboratory test results showed that white blood cells and hemoglobin were normal. Biochemical tests demonstrated the presence of glutamate-pyruvate transaminase at 446.5 IU/L (normal range, 0-40), glutamic-oxal (o) acetic transaminase at 277.3 IU/L (normal range, 5-34), alkaline phosphatase at 744.1 IU/L (normal range, 40-150), γ-glutamyltransferase at 1687.2 IU/L (normal range, 9-64), total bilirubin at 186.6 mg/dL (normal range, 3.4-20.5), direct bilirubin at 154.2 mg/dL (normal range, 0-8.6), and indirect bilirubin at 32.4 mg/dL (normal range, 3.4-11.9). The tumor markers of carcinoembryonic antigen were 4.74 ng/mL (normal range, 0-5), alpha fetoprotein 3.87 ng/mL (normal range, 0-9), and carbohydrate antigen 19-9 143.13 ng/mL (normal range, 0-37). Endoscopic ultrasound (Figure 1) showed a heterogenic, hypoechoic mass with ill-defined margins at the junction of the common bile duct (CBD) and the main pancreatic duct (PMD)- ampulla of Vater.

Conventional gray-scale ultrasound using a Logiq 9 scanner (GE, USA) equipped with a C2-4 transducer with a central frequency of 3.5 MHz revealed that the

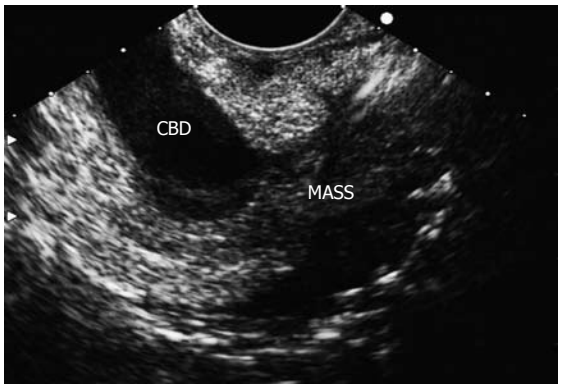

Figure 1 A heterogenic and hypoechoic mass (MASS) with ill-defined margin about 2.7 cm x 1.8 cm at the junction of the CBD and MPD.

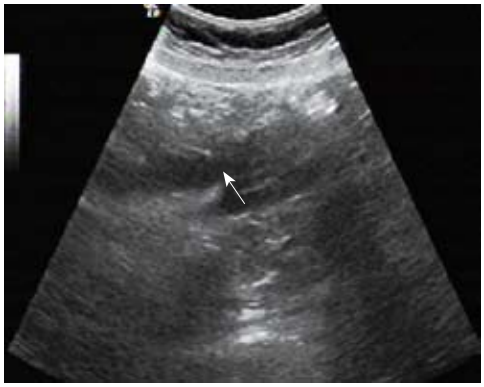

Figure 2 The end part of the CBD (arrow), which suddenly became narrow with a diameter of 0.7 cm. There was no exact mass detected.

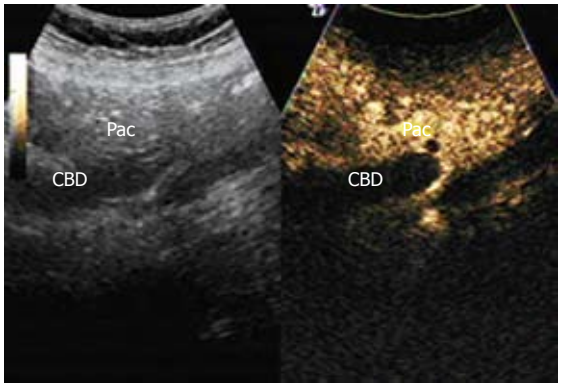

Figure 3 The wall of the CBD enhanced at 12 s after contrast agent was administered, while there was no obvious hyper-enhanced or hypo-enhanced lesion in the ampulla of Vater.

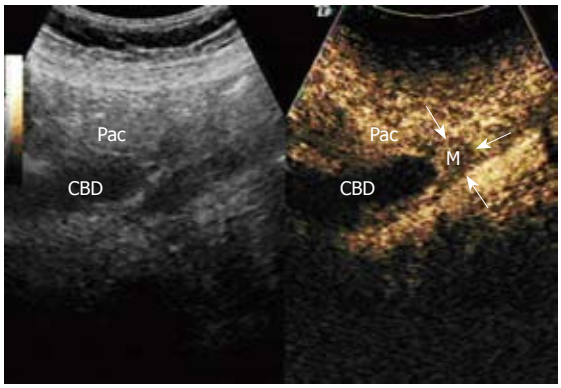

Figure 4 A hypo-enhanced lesion about 1.7 cm x 1.6 cm with blurred borders in the ampulla of Vater, from 20 s to 180 s, in comparison with the adjacent pancreas Pac.

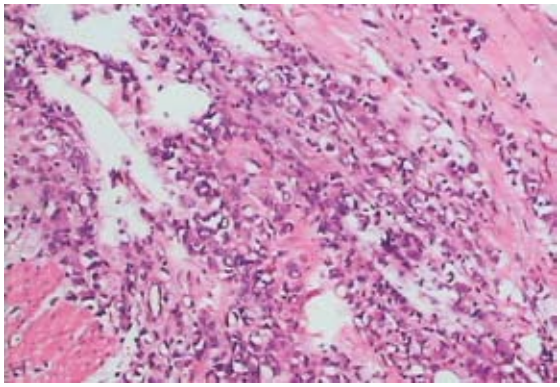

Figure 5 Micrograph shows SRCC of ampulla of Vater (HE x 100).

intrahepatic bile duct was dilated with a diameter of 0.8 cm, and the initial and intermediate portion of the CBD were dilated with a maximal diameter of 2.0 cm. The end part of the CBD suddenly became narrow, with a diameter of 0.7 cm, and there was no exact mass at the end of the CBD (Figure 2). CEUS was performed with low acoustic power, providing real-time imaging using low-mechanical index modes. Contrast-specific CEUS mode of contrast pulse sequencing was applied. The contrast agent, SonoVue (Brocca, Milan, Italy) (2.4 mL) was administered. The wall of the CBD began to enhance at 12 s after contrast agent was administered (Figure 3), while there was no obvious hyper-enhanced or hypo-enhanced lesion in the ampulla of Vater. A hypo-enhanced lesion about 1.7 cm × 1.6 cm with blurred borders in the ampulla of Vater was found from 20 s to 180 s, compared with the adjacent pancreas (Figure 4). At delayed phase (120 s after contrast agent administration), we scanned the whole liver and no abnormal enhanced lesions were found, indicating that there was no metastases in the liver.

The patient underwent a pancreato-duodenectomy with an extended lymphadenectomy and gastrectomy of 1/4 of the normal stomach. The mass was located in the ampulla of Vater with a size about 2.0 cm × 2.0 cm, brittle and protruding to the cavity of the duodenum. It had infiltrated to the periphery pancreatic tissue and adhered to the inferior vena cava. Lymph nodes of No. 16 were tumescent. The final pathological examination (Figure 5) showed that the cancer cells were widespread and polygonal, and the nuclei of the cells were located on one side, which are prominent signet-ring features. Final pathology confirmed an SRCC of the ampulla of Vater, and the pancreas and the whole wall of the duodenum were infiltrated with carcinoma. No distal or nodal metastases were identified. There was no evidence of lymphatic and vascular invasion. The ampullary cancer was

diagnosed as T3N0M0, stage Ⅱ A according to American Joint Committee on Cancer TNM classification[1] and International Union Against Cancer TNM classification[2].

Our patient did not receive adjuvant therapies such as chemotherapy or radiotherapy after the operation. The patient remained well and had no evidence of recurrence and distant metastases during the 6-mo follow-up.

DISCUSSION

SRCC can arise in many organs, but it usually occurs in the gastrointestinal tract, especially in the stomach. It has been reported that 90% of SRCC occurs in the stomach, with the rest arising in several other organs, including the breast, gallbladder, pancreas, urinary bladder and colon[3]. It is extremely uncommon in the ampulla of Vater. Less than 15 cases have been described in the literature. Akatsu[4] has summarized the previous 14 cases (eight men and six women) and concluded that the median age at diagnosis was 57 years (range, 32-83 years), approximately 15 years older than SRCC of the stomach, but similar to the median age for SRCC of the large bowel. Our case was a 38-year-old woman. It is very rare[5]. The origin for SRCC of the ampulla of Vater remains controversial; one theory is that these tumors may originate from heterotopic gastric mucosa. Another theory holds that these carcinomas arise from areas of gastric-type metaplastic epithelia, which are considered to be a protective response to elevated acidity and are observable in the duodenal bulb of peptic ulcer patients[6]. However, there was no history of peptic ulcer disease in our patient and no ectopic gastric epithelium was found in the tumor and peritumoral tissues. Surgery is the the first choice for the treatment of such disease. As for the prognosis, poorly differentiated adenocarcinoma is more frequently associated with an advanced tumor stage and poor prognosis in cases of ampullary carcinoma. SRCC elsewhere in the gastrointestinal tract has a poor prognosis. However, it is unclear whether the prognosis of a patient with SRCC is worse than that of patients with ordinary carcinoma occurring in the ampulla of Vater, because of the small number of cases so far reported. A patient has been reported to survive for 7.5 years after radical resection, and the author has suggested that long-term survival is possible in ampullary SRCC without nodal involvement[4]. A 58-year-old patient lived for 134 mo after resection and had no evidence of recurrence[7].

As for diagnosis of SRCC, helical computed tomography (CT) only showed a dilated CBD without a mass lesion in the ampulla of Vater in some cases[5,7-9]. Upon ultrasound, it may present as an abnormal echoic mass in the ampulla of Vater[6], or obstruction and dilation of the CBD at the level of the pancreas[8,9]. Our case is unique because we underwent a special examination with CEUS before surgery and found the lesion, which was not discovered by conventional gray-scale ultrasound.

CEUS has gained increasing interest in recent years. The properties of SonoVue and the high sensitivity of recent ultrasound equipment to the presence of microbubbles have shown that CEUS is potentially very useful in revealing many organs and vascular structures. It has been a rapidly evolving technique for clinical application. CEUS allows the assessment of the macrovasculature and microvasculature in different parenchymas, and the identification and characterization of lesions in organs. It has been reportes that CEUS produced results very similar to those obtained with contrast-enhanced CT and magnetic resonance imaging in the characterization of various liver lesions. The causes of obstructive jaundice can be divided into two categories: tumorous and non-malignant stenosis. For non-malignant stenosis, such as acute or chronic inflammation of the papilla, fibroid stenosis at the end of the CBD can be irritated by cholesterol calculi or sludge at the end of the CBD; blood clots at the end of the CBD may cause obstructive jaundice too. However, the main cause of obstructive jaundice is the tumors arising from the ampulla of Vater, and mostly are malignant. In the diagnosis of obstructive jaundice, the emphasis should be laid on excluding the non-malignant reasons: non-shadowing stones, blood clots and sludge. This may influence the selection of therapy. The non-shadowing stones, blood clots and sludge may appear non-enhanced by CEUS because of an absence of blood supply. In the present case, the carcinoma showed iso-enhancement at an early stage after contrast agent administration, and obvious hypo-enhancement at the delayed phase, because it had intravital tissue with blood supply; microbubbles are distributed within the blood and appear wherever there is a blood supply. Our case showed that CEUS may provide an effective means of diagnosis of ampullary carcinomas. CEUS could offer real-time imaging of the microcirculation in the lesions. By CEUS, the lesion may be displayed much clearer than by conventional gray-scale ultrasound. It can also offer a good method in the discrimination of ampullary carcinoma from non-malignant lesions.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

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

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

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

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

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

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

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

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

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

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

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Instructions to authors

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension,

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

Both personal authors and an organization as author

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

No author given

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

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

Issue with no volume

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

No volume or issue

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

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Personal author(s)

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

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

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

Conference proceedings

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

Conference paper

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

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/eid.htm

Patent (list all authors)

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

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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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
Biology: *H pylori*, *E coli*, *etc*.

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