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The WJG Press, Apartment 1066 Yishou Garden, 58 North
Langxinzhuang Road, PO Box 2345, Beijing 100023, China
Telephone: +86-10-85381901
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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Telephone: +86-10-85381901
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

SUBSCRIPTION AND AUTHOR REPRINTS
Jing Wang
The WJG Press, Apartment 1066 Yishou Garden, 58 North Langxinzhuang Road, PO Box 2345, Beijing 100023, China
Telephone: +86-10-85381901
Fax: +86-10-85381893
E-mail: j.wang@wjgnet.com
http://www.wjgnet.com

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REVIEW

Gut hormones, and short bowel syndrome: The enigmatic role of glucagon-like peptide-2 in the regulation of intestinal adaptation

GR Martin, PL Beck, DL Sigalet

GR Martin, PL Beck, DL Sigalet, University of Calgary, Gastrointestinal Research Group, Calgary, Alberta T2N 4N1, Canada
Supported by the Alberta Children's Hospital Research Foundation
Correspondence to: Gary Martin, Department of Gastrointestinal Sciences, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW., Calgary, Alberta T2N 4N1, Canada. marting@ucalgary.ca
Telephone: +1-403-2203189 Fax: +1-403-2833840
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Abstract

Short bowel syndrome (SBS) refers to the malabsorption of nutrients, water, and essential vitamins as a result of disease or surgical removal of parts of the small intestine. The most common reasons for removing part of the small intestine are due to surgical intervention for the treatment of either Crohn's disease or necrotizing enterocolitis. Intestinal adaptation following resection may take weeks to months to be achieved, thus nutritional support requires a variety of therapeutic measures, which include parenteral nutrition. Improper nutrition management can leave the SBS patient malnourished and/or dehydrated, which can be life threatening. The development of therapeutic strategies that reduce both the complications and medical costs associated with SBS/long-term parenteral nutrition while enhancing the intestinal adaptive response would be valuable.

Currently, therapeutic options available for the treatment of SBS are limited. There are many potential stimulators of intestinal adaptation including peptide hormones, growth factors, and neuronally-derived components. Glucagon-like peptide-2 (GLP-2) is one potential treatment for gastrointestinal disorders associated with insufficient mucosal function. A significant body of evidence demonstrates that GLP-2 is a trophic hormone that plays an important role in controlling intestinal adaptation. Recent data from clinical trials demonstrate that GLP-2 is safe, well-tolerated, and promotes intestinal growth in SBS patients. However, the mechanism of action and the localization of the glucagon-like peptide-2 receptor (GLP-2R) remains an enigma. This review summarizes the role of a number of mucosal-derived factors that might be involved with intestinal adaptation processes; however, this discussion primarily examines the physiology, mechanism of action, and utility of GLP-2 in the regulation of intestinal mucosal growth.

Key words: Short bowel syndrome; Glucagon-like peptide-2; Epidermal growth factor; Insulin-like growth factor-I; Parenteral nutrition; Total parenteral nutrition; Intestinal adaptation; Intestinal mucosa; Gut hormones; Enteric nervous system

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INTRODUCTION

Advances in surgical and medical care have made it possible for those with massive small bowel resections to survive. Unfortunately, a loss of greater than 50% of the small intestine often results in short bowel syndrome (SBS)[1-4]. SBS is characterized by the inefficient absorption of nutrients and fluids resulting in severe diarrhea, dehydration, electrolyte imbalances, nutrient deficiencies, weight loss, and frequently, a long-term dependence on parenteral nutrition[4-6]. In the pediatric population, total parenteral nutrition (TPN) maintenance-associated complications frequently result in sepsis, secondary liver failure, and high morbidity[5,7,8]. The development of therapeutic strategies that could reduce the consequences of SBS and long-term parenteral nutrition while enhancing the intestinal adaptive response would be valuable.

Numerous non-nutritional factors are potential stimulators of intestinal adaptation including peptide hormones, growth factors, and neuronally-derived components[9]. Some of the growth factors that have been shown to stimulate intestinal growth include glucagon-like peptide-2 (GLP-2), epidermal growth factor (EGF), growth hormone (GH), insulin-like growth factor-1 (IGF-1), neurotensin (NT), intestinal trefoil factor (ITF), transforming growth factor (TGF-α; TGF-β), and

Table 1 Growth factors as potential stimulators of intestinal adaptation

Mucosal growth factors and SBS			
Growth factor	**Amino acids /MW**	**Main source**	**GI receptor localization**
GLP-2	33 aa/3.8 kDa	Ileal and colonic EE L-cells, pancreatic α cells	GLP-2R; EE cells, myenteric/submucosal neurons of ENS
EGF	53 aa/6.4 kDa	Submandibular gland, duodenal Brunner's gland, epithelial and Paneth cells	EGFR/HER/ErbB, HER2/Neu/ErbB2, HER3/ErbB3, HER4/ErbB4
KGF (FGF-7)	194 aa/2.5 kDa	Fibroblast-like stromal cells in epithelial tissue	KGFR; a/bFGFR epithelial cells
Hb-EGF	86 aa/22 kDa	Smooth muscle cells	Activate all as listed for the EGFR
GH	191 aa/22 kDa	Liver	CRF type-1, PRL-R
TGF-α	50-53 aa/6.2-6.4 kDa	Epithelial cells	Activate all as listed for the EGFR
NT	13 aa/?	Ileum/colon >> proximal SB, EE N-cells	NTR1
PYY	36 aa/42 kDa	Ileal and colonic EE L-cells	Y1-R; EE cells, myenteric/submucosal neurons of ENS
GRP	14 aa/?	Neuroendocrine cells in the ENS, fibroblasts	GRPR antrum, upper GI tract
CCK	8, 33 aa/39 kDa	Duodenal EE I-cells	CCK_A >> CCK_B upper GI tissue myenteric/submucosal neurons of ENS
Gastrin	17 aa/21 kDa	Gastric antrum G-cells	CCK_B >> CCK_A
	34 aa/40 kDa	Pancreatic D-cells	upper GI tissue
IGF-I/II	I-70 aa/7.6 kDa, II-67 aa/7.4 kDa	Proximal SB >> distal, liver, Fibroblasts	IGF-IR and IGF-IIR epithelial cells, glial cells, fibroblasts

Many of these factors include peptide hormones, growth factors, and neurovascular components. SB, small bowel; ENS, enteric nervous system; EE, enteroendocrine; GLP-2, glucagon-like peptide-2; EGF, epidermal growth factor; Hb-EGF, heparin-binding EGF; KGF, keratinocyte growth factor (FGF-7); FGF, fibroblast growth factor; GH, growth hormone; CRF-1, cytokine-related family type-1 (JAK-STAT pathway); PRL, prolactin (subgroup of the CRF-1 superfamily); TGF-α, transforming growth factor-α; NT, neurotensin; PYY, peptide tyrosine tyrosine; GRP, gastrin-releasing peptide; CCK, cholecystokinin; IGF-I/II, insulin-like growth factor I/II.

fibroblast growth factor (FGF)[10]. Table 1 lists some of the mucosa-derived humoral factors that have been shown to promote intestinal adaptation following intestinal resection.

GLP-2 is a potential therapy currently under consideration for the treatment of gastrointestinal disorders that are associated with insufficient mucosal function. Evidence from both animal studies and clinical trials demonstrate that GLP-2 is a trophic hormone that plays an important role in the regulation of intestinal adaptation[11]. Under normal conditions, GLP-2 is secreted into the circulation in response to the ingestion of a mixed meal[12-15], particularly following one that contains carbohydrates, fatty acids, and fiber[16-18]. These studies have shown that following nutrient-induced release or exogenous administration, GLP-2 is involved in the regulation of cell proliferation, apoptosis, nutrient absorption, motility, as well as epithelial and intestinal permeability[15,19-25].

Initial pilot Phase II trials in SBS patients treated with teduglutide, a GLP-2 analog, observed that there were modest increases in fluid and nutrient absorption. Based upon the positive results of this trial, Phase III studies in adults with SBS were initiated. Subsequently, a recent article in *GUT* shows that teduglutide, using various dosing modalities over a 21 d experimental period, was safe, well tolerated, and promoted intestinal growth in SBS patients having either an end jejunostomy or a colon in continuity[26]. Moreover, teduglutide therapy in these SBS patients resulted in a significant increase in small intestinal villus height, crypt depth, and mitotic index. The authors state that the most significant clinical benefits of teduglutide in treating SBS patients were the reduction of intestinal wet weight excretion and in improving wet weight absorption[26]. There are several other clinical trials underway that are examining potential therapeutics for the treatment of SBS, unfortunately, Zorbitive® (hGH, glutamine) remains the only option presently available for prescriptive use.

This review summarizes the current understanding of a number of growth factors and their role in the treatment of SBS. As it is unlikely that there is just one specific growth factor responsible for maintaining mucosal growth, an important consideration is that there is likely some degree of regulation, transactivation, and crosstalk, between established stimulators of mucosal growth (i.e. epidermal growth factor, insulin-like growth factor-I, polyamines, GLP-2). Thus an understanding of the collective influence of these promoters of intestinal adaptation should not be dismissed. However, the primary focus of this discussion is to examine the physiology, mechanism of action, and utility of GLP-2 in the regulation of intestinal mucosal growth.

Discovery of GLP-2

The first indication that GLP-2 was trophic to the bowel was the observation that patients with glucagonomas had significantly elevated circulating enteroglucagon levels that was secondarily associated with intestinal mucosal hyperplasia[27,28]. The correlations between serum enteroglucagon levels and mucosal growth of the small intestine provided further evidence for the trophic effects of GLP-2[27]. A major breakthrough came in 1996 when it was shown that injection of proglucagon-producing tumors into nude mice resulted in significant mucosal hyperplasia[29]. Isolation of the various components of these proglucagon-producing tumors revealed that GLP-2 was the trophic factor responsible for the increased

mucosal growth.

Additional evidence for intestinotrophic properties of GLP-2 came from studies in rats and mice in which GLP-2 administration induced intestinal hypertrophy i.e., significant increases in protein and DNA content, villus height, and bowel weight[21,25,30,31]. Moreover, GLP-2 treatment reduced mucosal atrophy associated with TPN administration and augmented the adaptive growth response following massive small bowel resection[19-21,32]. Our studies showed that in parenterally maintained animals (no luminal nutrition), infusion with GLP-2 stimulated intestinal adaptation following small bowel resection[18]. Without the infusion of GLP-2, intestinal adaptation did not occur. Similarly, the immunoneutralization of endogenous circulatory GLP-2 reduced the intestinal post-resection adaptive response in rats and rabbits[33].

What is GLP-2?

GLP-2 is a member of the PACAP (Pituitary adenylate cyclase activating peptide)/Glucagon superfamily that includes the enteroglucagon/proglucagon-derived peptides (PGDP) that are produced in the L-cells of the small intestinal and colonic mucosa. The L-cells have been identified as the cellular origin of enteroglucagon (Figure 1)[24,34,35]. In 1983, the full sequence of hamster proglucagon, and later that year, the human sequence, were published revealing the sequences of GLP-1 and GLP-2[36,37]. GLP-2 is described as glucagon-like as it shares approximately 50% sequence homology with glucagon[37]. Sequencing of GLP-2 from human and pig intestine has confirmed the structure of GLP-2 as a 33 amino acid peptide that contains alanine at position 2 of the N-terminus (making it easily degraded by exopeptidases) and ends with a carboxy-terminal Asp residue[45]. GLP-2 and the other PGDPs are derived following the proteolytic cleavage and other enzymatic modifications of proglucagon in the organelles of the pancreatic α-cells and intestinal L-cells[38,39]. Interestingly, GLP-2 and the other products of post-translational processing (glicentin, GLP-1, oxyntomodulin) are produced in the intestinal L-cells, while glucagon and the major proglucagon fragment (MPGF) are produced in the α-cells of the pancreas[40]. They are formed from the same precursor molecule and therefore must be secreted in parallel. Differential expression of prohormone convertase (PC), the enzyme responsible for cleaving proglucagon into various hormonal products in the different cells, accounts for the different products. The PC2 enzyme, which has a greater abundance in the pancreatic α-cells, preferentially produces glucagon as its end product[41-43]. On the other hand, the PC1/3 enzyme, which is found in greater abundance in the intestinal L-cell, predominantly generates the glucagon-like peptides (GLP-1/2)[41]. Therefore, the differential processing of proglucagon in pancreatic α-cells by PC2, and by PC1/3 in the intestinal L-cell, might generate two unique hormonal products from the same precursor with each having a different physiological effect[43].

Initially, the GLP-2 sequence was identified in all mammalian cDNA, but not in other species. This led researchers to theorize that GLP-2 was a late evolutionary addition. Subsequent studies have demonstrated that fish, chickens, and lizards generate GLP-2 in the gut as a result of tissue-specific alternative RNA splicing of proglucagon RNA transcript[44].

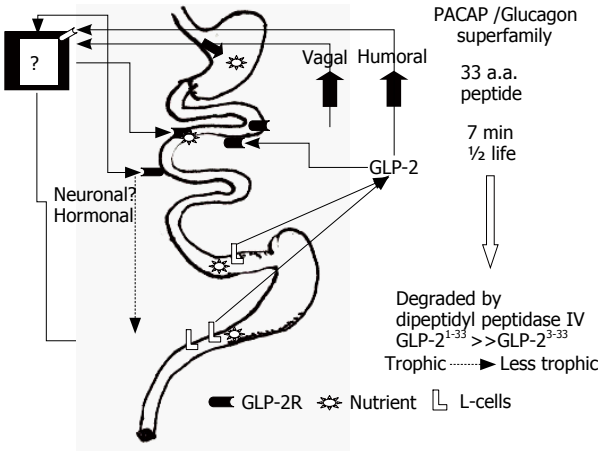

Figure 1 Potential pathways involved in the production, release, activity, and sites of action of GLP-2. GLP-2 produced by the L-cell is induced following the ingestion of a meal and can be induced either by direct stimulation of the L-cell, or potentially following the release of upstream neuronal /hormonal agonists. The GLP-2R has been localized to components of the enteric nervous system (ENS) and to regions of the brain, thus the growth-promoting effects associated with GLP-2 may involve interactions with the brain-gut axis. Potentially the ENS / vagal interface might be responsible for the early release of GLP-2.

Site of GLP-2 production

The distal regions of the small bowel and/or the large intestine are necessary for GLP-2 production. This was demonstrated in a study in which patients with SBS, without a colon in continuity, had significant reductions in their post-prandial circulatory GLP-2. Subjects that had 140 cm or less of small bowel, with colon in continuity, exhibited a significant increase in their serum GLP-2 levels[46,47]. Conversely, fasting and post-prandial serum GLP-2 levels were elevated in SBS patients (colon intact, no ileum) compared with sex- and age-matched controls[46]. The locale of the GLP-2-containing tissue in the human intestine is debatable, though it was recently reported that the number of immunoreactive GLP-2 cells increase in a proximal to distal distribution in normal colonic tissue[48].

However, there is debate as to the contribution of different regions of the bowel to total GLP-2 production. A 90% intestinal resection, leaving only 10 cm of distal ileum, results in a significant elevation in serum GLP-2 levels in the rat[15]. These elevations in circulatory GLP-2 correlate with several measurable parameters of intestinal adaptation[18]. As the remainder of the small bowel had been removed, this suggests that the distal ileum and/or the colon are the major sites of the L-cells, the cells responsible for the production of GLP-2 (Figure 1). Others describe that in the rat, TNBS (trinitrobenzene sulphonic acid) -induced ileitis resulted in no change in the number of GLP-2 immunoreactive cells in the ileum, yet colonic GLP-2 immunoreactivity was increased (personal communication, Jennifer O'Hara and Dr. K.A. Sharkey).

Interestingly, in a rat SBS model in which only 20 cm of jejunum was anastomosed directly to the ascending colon[49] (i.e., removal of 80% of the distal small bowel

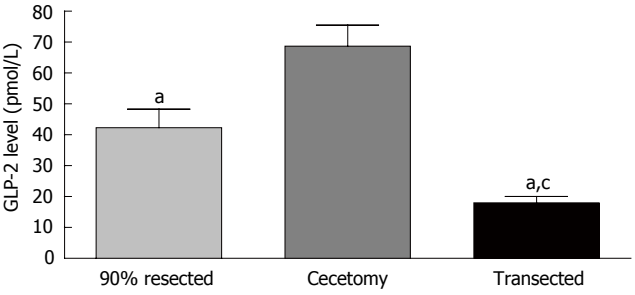

Figure 2 An experimental rat model of SBS-induces changes in circulatory GLP-2 levels. The distal regions of the small bowel and/or the large intestine are necessary for GLP-2 production. In these experiments, the entire proximal small bowel was removed; therefore the distal ileum and/or the colon are likely the cells responsible for the production of GLP-2 as all other bowel tissue had been removed. Resected = 90% intestinal resection leaving a 10 cm ileal remnant. CEC = cecetomy + removal of all but 20 cm of jejunum. The remnant jejunum was anastomosed to the ascending colon. $^{a}P < 0.05$ *vs* cecetomized animals; $^{c}P < 0.05$ *vs* 90% proximal resected animals that were enterally-fed (10 cm remnant ileum).

including the ileum and cecum), circulatory GLP-2 levels were significantly increased in comparison to both controls and the 90% resection animals with ileum intact (Figure 2). This strongly suggests that the colon is an important source of GLP-2. These findings emphasize the importance of determining if the increase in serum GLP-2 levels are the result of either an increase in the L-cell population, or alternatively, as a consequence of augmented GLP-2 production by the L-cells. Our ongoing experiments suggest, at least in this rat model of SBS, that increased GLP-2 production is the result of an increase in the colonic L-cell population (unpublished observations).

GLP-2 receptor and activity

Currently, the localization of the glucagon-like-2 receptor (GLP-2R) remains an enigma. Conflicting data in the literature report the GLP-2R to be located on enteroendocrine cells[50], on non-epithelial elements such as neuronal cells[51], and subepithelial myofibroblasts (Figure 1)[52]. Moreover, the localization of the GLP-2R in the bowel might be region-specific. In a recent study by Orskov *et al*, GLP-2 receptors were found throughout the small and large bowel in humans, mice, marmoset, and rat[52]. Furthermore, the intestinal region that had the greatest GLP-2R immunoreactivity was the proximal small bowel. These data were confirmed by both immunohistochemistry and *in situ* hybridization that showed that the GLP-2R was localized to cells lying beneath the basal membrane of enterocytes[52].

The specific GLP-2 receptor has been isolated, sequenced, and found to be expressed in both the gut and in the hypothalamus (Figure 1)[53]. GLP-2 binds to a 7 transmembrane (7-TM) G protein-coupled receptor that is comprised of 550 amino acids. In humans, the expression of the GLP-2R is localized to specific enteroendocrine cells in the stomach as well as the small and large bowel[53-55]. Currently there have been only two human cervical carcinoma cell lines, including HeLa cells, in which GLP-2R mRNA transcripts have been detected[56]: the majority of the signal pathway determinations have been carried out in cells transfected with the GLP-2R[55]. The

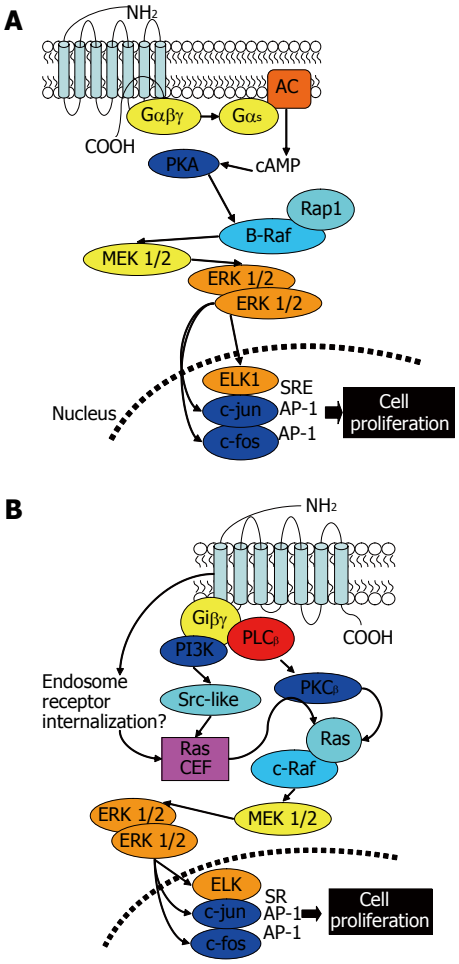

Figure 3 **A**: GLP-2 stimulation of the G_s-Coupled Receptor induced cell proliferation. The proposed GLP-2 /GLP-2R signaling pathway as characterized in cells that were transfected with the GLP-2R. See section on GLP-2 receptor-activity for description (pages 11-12); **B**: GLP-2 stimulation of the $G\alpha_i$-Coupled Receptor induced cell proliferation. Possible trafficking pathways triggered by GLP-2 in cells that tested negative for the characterized GLP-2R. Potentially, GLP-2 induces divergent signaling pathways which results in an increase in the rate of cell proliferation. See section on GLP-2 receptor-activity for description (pages 11-12).

enteroendocrine cells that do express the GLP-2R have also been found to express other gut hormones such as glucagon-like peptide-1, glucose dependent insulinotropic peptide, peptide YY, and serotonin[50]. PACAP and VIP share the same $VPAC_1$ receptor with equal affinity[34], thus it might be possible that other members of this family also share receptors.

There are a number of unanswered questions regarding the signaling mechanism involved in how GLP-2 stimulates mucosal growth. The initial studies on intracellular trafficking was done entirely in cell lines (BHK /COS) that had been transfected with the characterized GLP-2R[55,57]. These data showed that GLP-2/GLP-2R signaling involved classic G protein-coupled $G\alpha_s$ activation of adenylyl cyclase (AC), increased cAMP and PKA accumulation, and eventually ELK-1/c-fos/c-jun gene activation and increased cell proliferation (Figure 3A). Conversely, in epithelial cell lines devoid of the GLP-2R (as confirmed by both Western blot analysis and by the absence of mRNA transcripts), GLP-2 administration was still capable

of increasing cell proliferation[56,58,59]. Thymidine uptake as a measure of an increased proliferation was significantly inhibited following pre-incubation with pertussis toxin ($G\alpha_i$ inhibitor) or MAPK blockade, implying that $G\alpha_i$ or EGFR/TyrK trafficking pathways are involved (Figure 3B). These studies also revealed that, in comparison with the transfected cell lines in which increases in cAMP correlated with augmented growth, there was a significant correlation between increased cell proliferation and decreased cAMP accumulation.

Furthermore, the possibility that there are multiple signaling mechanisms for GLP-2 is suggested by recent observations regarding the divergent effects of GLP-2 administration on epithelial cell proliferation. These effects were dependent on both the cell line and the dose of GLP-2 administered. Two independent studies demonstrated that administration of GLP-2 results in the inhibition of proliferation of non-transformed small intestinal epithelial cell lines (IEC-6, IEC-18), while increasing proliferation of cancer-derived human colonic epithelial cell lines (Caco-2, Colo 320)[59,60]. Moreover, there can be divergent effects of GLP-2 on epithelial cell migration, as GLP-2 significantly increased migration in the small intestine-derived IEC-6/IEC-18 cells, but had no effect on epithelial cell migration in the colonic Caco-2 or the Colo 320 cells[60].

An article recently published in *Science* may have uncovered another important component of the GLP-2 signaling pathway[61]. It is established that the intestinal epithelium undergoes constant renewal along the crypt-villus axis and that the β-catenin/T cell factor (TCF) transduction pathway is involved in these proliferation/differentiation events[62-64]. These data reveal that GLP-2 treatment of mice increases β-catenin levels suggesting that β-catenin might be a downstream mediator of GLP-2-induced crypt cell proliferation. In addition, the expression of a cDNA encoding human R-spondin1 (hRSpo1) in KI chimeras led to a substantial increase in the diameter, length, and weight of the small intestine, diffuse thickening of the mucosa, and crypt epithelial hyperplasia[61]. Their data showed that the administration of hRSpo1 potently affects proliferation of the intestinal epithelium through activation of β-catenin; the activation of this pathway by hRSpo1 indicates that the effect might be directly stimulated by receptor-mediated binding. It is interesting that the expression of hRSpo1 in human intestinal enteroendocrine cells, consistent with the role of this protein as a crypt cell mitogen, is similarly localized to the expression of GLP-2[61]. This is certainly an exciting find in regards to mapping out potential signaling pathways involved in GLP-2-induced intestinal growth.

Collectively, this dichotomy suggests that there are divergent signaling pathways involved in how GLP-2 stimulates cell proliferation. Secondly, it begs the question: are other uncharacterized GLP-2R's or transactivation signaling mechanisms involved in the intestinotrophic effect of GLP-2 other than that initially described by Munroe *et al*[53]?

GLP-2 interactions with the ENS/CNS

There is evidence that the GLP-2R is expressed on both murine enteric neurons and cerebral cortex astrocytes[51,65]. Bjerknes and Cheng showed by *in situ* hybridization that the GLP-2R is located on nonepithelial elements of the enteric nervous system. These results were further verified by reverse-transcriptase polymerase chain reaction (RT-PCR) for the GLP-2R mRNA[51]. Moreover, the GLP-2R was located on neurons and not on glial cells. These experiments demonstrate that GLP-2 is capable of inducing *c-Fos* activity in enteric neurons in as little as 7 min, and achieved maximal expression by 15 min. After 90 min, the increase in GLP-2-induced neuronal *c-Fos* activity was followed by increased *c-Fos* activity in the crypt cells of the jejunum and colon. These observations suggest that GLP-2 stimulates the GLP-2R leading to an increased *c-Fos* expression in myenteric neurons (Figure 4), which is secondarily followed by both an increase in *c-Fos* expression in the crypt cells and the stimulation of columnar crypt cell growth. Tetrodotoxin (TTX) pre-treatment abolished *c-Fos* expression in the crypt cells revealing that GLP-2 signaling in the bowel might be dependent on a transduction pathway requiring enteric neurons[51]. Potentially the intestinotrophic property associated with GLP-2 involves a second downstream mediator that is released upon GLP-2's initial stimulation of the ENS (Figure 4).

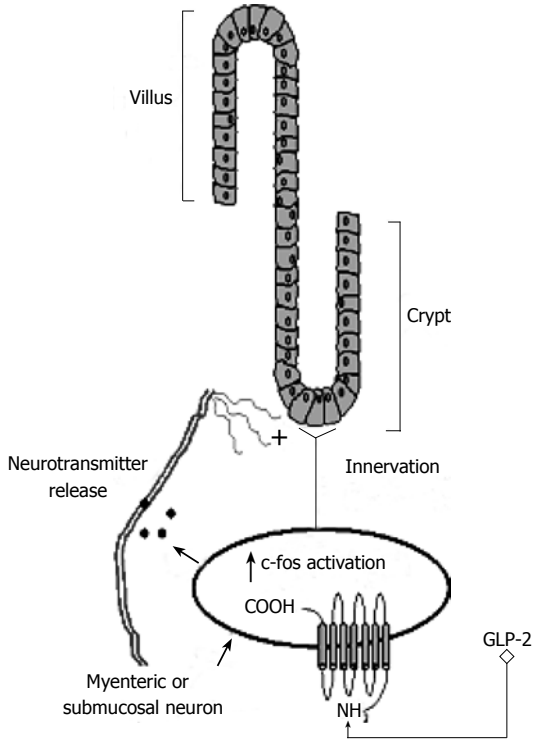

Figure 4 GLP-2 stimulation of the enteric nervous system. GLP-2 binds to 7-TM G protein-coupled receptors localized to components of the ENS. This results in a conformational change in the GLP-2R that eventually activates c-fos expression via the Gαs, AC, c-AMP pathway. This suggests that a rise in circulatory GLP-2 levelsmight stimulate the GLP-2R leading to an increased stimulation of the myenteric and/or submucosal neurons. Potentially the trophic effects on the gut mucosa associated with GLP-2 are the result of a second downstream mediator, either a neurotransmitter or direct innervation that is activated upon GLP-2's initial stimulation of the ENS localized GLP-2R.

The GLP-2R is also localized to regions of the hindbrain[66]. The dorsal medial hypothalamus (DMH) contains a dense plexus of GLP-2 immunoreactive fibers that express GLP-2 receptor mRNA (Figure 1). Though

research regarding GLP-2's involvement in neuronal activation is still in its infancy, it is interesting that GLP-2's effects on enteric /vagal pathways are remarkably similar to those characterized for cholecystokinin[67,68]. This possibility has led to discussions regarding GLP-2 as a factor involved in the regulation of feeding. There is evidence that GLP-2 administration can inhibit feed intake[66,69,70] however, other investigators report no effect on appetite or energy intake at physiological concentrations of GLP-2[71]. This area of GLP-2 physiology requires further investigation.

These observations suggest a novel pathway of GLP-2 action as perhaps either a meal-stimulated endogenous release of GLP-2 from the L-cell, or potentially, the therapeutic administration of GLP-2, could activate enteric neuronal activity, resulting secondarily in the release of downstream mediators capable of inducing crypt cell proliferation (Figure 4). Currently, no studies have been published in which the expression of the GLP-2R in the human enteric nervous system was examined.

GLP-2 receptor antagonists

An obstacle when examining mechanistic pathways involved in GLP-2-induced small bowel adaptation is the lack of effective GLP-2R antagonists. One possibility would be to use the primary degradation product ($GLP\text{-}2^{3\text{-}33}$) to see if the interaction with the GLP-2R could reduce the adaptive response to intestinal resection. These results might be difficult to interpret as the treatment of mice with either $GLP\text{-}2^{1\text{-}33}$ or $GLP\text{-}2^{3\text{-}33}$ induces significant growth in both the small and large bowel, though the growth response induced by the degradation product is reduced[72]. Furthermore, functional studies and binding data indicate that $GLP\text{-}2^{3\text{-}33}$ acts as both a partial agonist and as a partial antagonist on the GLP-2R[72]. However, there is recent data that demonstrates $GLP\text{-}2^{3\text{-}33}$ antagonizes the growth-promoting activities of $GLP\text{-}2^{1\text{-}33}$ *in vitro* and *ex vivo*[73]. Shin *et al* suggest that endogenous $GLP\text{-}2^{1\text{-}33}$ regulates the intestinal growth-promoting response via modulation of crypt-cell proliferation and villus apoptosis[73]. Collectively, these findings do not support the use of $GLP\text{-}2^{3\text{-}33}$ as a full antagonist of the GLP-2 receptor.

GLP-2 inactivation and metabolism

As mentioned earlier in this review, $GLP\text{-}2^{1\text{-}33}$ contains the amino acid alanine at position 2 thereby making it susceptible to degradation by the exopeptidase dipeptidyl peptidase IV (DPP-IV)[74]. For excellent reviews on the structural and functional properties of DPP-IV, see Mentlein or Lambeir[75,76]. The primary metabolism of $GLP\text{-}2^{1\text{-}33}$ by DPP-IV, which cleaves off two N-terminal amino acids, results in the formation of $GLP\text{-}2^{3\text{-}33}$, a potential receptor antagonist /agonist[72,73]. The elimination half-life of intact $GLP\text{-}2^{1\text{-}33}$ in humans is 7 min, whereas that of cleaved $GLP\text{-}2^{3\text{-}33}$ is 27 min[14], thus the cleavage of the NH_2 terminal may potentially disrupt the signal transduction pathway and reduce GLP-2's biological effectiveness. The administration of GLP-2 to DPP-IV deficient rats results in higher $GLP\text{-}2^{1\text{-}33}$ serum levels which correlate with a significant increase in small intestinal weight[74,77]. Similar increases in intestinal hypertrophy also occur in rats and mice administered a synthetic DPP-IV resistant GLP-2 analog (ALX-0600)[31,74] suggesting that the inactivation of $GLP\text{-}2^{1\text{-}33}$ by DPP-IV is a crucial limiting factor regarding GLP-2's trophic effect on the gut mucosa. In humans, the replacement of alanine in position 2 of the GLP-2 peptide with glycine, has been shown to extend the half life from approximately 7 min, as for GLP-2, to 0.9-2.3 h.[26] Thus, DPP-IV resistant GLP-2 analogues might be useful therapeutically to help improve mucosal regeneration.

In contrast, there is evidence that DPP-IV may not be the only factor important in the degradation of GLP-2[78]. Geier *et al* did not detect an observable role for DPP-IV in the regulation of GLP-2 in $DPP\text{-}IV^{-/-}$ mice, as there were no measurable differences in GLP-2 levels or plasma DPP-IV-like activity between $DPP\text{-}IV^{+/+}$ and $DPP\text{-}IV^{-/-}$ mice. Although $DPP\text{-}IV^{-/-}$ mice lack the *DPP-IV* gene, they do possess the genes for FAP, DP8, and DP9, and might have sufficient dipeptidyl peptidase levels to regulate GLP-2 cleavage. Moreover, the mice lacking the *DPP-IV* gene do not have either intrinsic resistance, or an enhanced rate of repair, in DSS-induced colitis. The authors concluded that residual dipeptidyl peptidase levels in the $DPP\text{-}IV^{-/-}$ mice results in insufficient $GLP\text{-}2^{1\text{-}33}$ bioavailability to protect these mice from DSS (dextran sodium sulfate) -induced damage The association between GLP-2 and DPP-IV family members will require further study.

Plasma concentrations of GLP-2 are not only influenced by the rate of secretion, but by the elimination or clearance rate. The kidney is important in the clearance of GLP-2 as there are increased circulatory levels in patients with renal failure[79,80]. In addition, experimental nephrectomy results in delayed clearance and increased circulating levels of GLP-2 in rats[81]. The increase in circulatory GLP-2 levels following nephrectomy is not surprising as it has been shown that the kidney is the site of greatest DPP-IV activity[75].

GLP-2 and intestinal inflammation

The treatment of animals with GLP-2 reduces harmful symptoms of inflammation associated with colitis as well as the mortality and the severity of both indomethacin and TNBS-induced enteritis[35,82,83]. Moreover, studies within our lab reveal that GLP-2 ameliorates inflammatory parameters associated with TNBS-induced ileitis (Gastroenterology abstract, T1530, 2005). These reductions in inflammation (decreased myeloperoxidase activity, interleukin-1β, and inducible nitric oxide synthase protein in mucosal tissue) 5 d post-TNBS occurred whether the GLP-2 was given simultaneously with TNBS, or 2 d post-TNBS injection. Others have observed that GLP-2 treatment initiated following chemotherapy enhances intestinal recovery[84]. A second report also showed the benefits of GLP-2 treatment as a preventative against cancer therapy-induced mucosal damage[85]. Teduglutide, a GLP-2 analog, given daily prior to whole body gamma-irradiation significantly increased crypt stem cell survival in mice when compared with vehicle-treated controls, though the protective effect was only observed when teduglutide was given prior to irradiation[85]. Collectively, these reports indicate that GLP-2 might be a useful therapeutic strategy in

the treatment of diseases associated with intestinal inflammation.

There are conflicting reports regarding the effect of intestinal inflammation on GLP-2 production, particularly on the site of GLP-2 production. Intuitively, any situation that depletes the GLP-2-producing L-cells could potentially reduce the ability of GLP-2 to repair the mucosa. This could be the case in Crohn's/colitis patients as inflammation of the intestine likely disrupts the L-cells. In a mouse-model of T-cell-induced inflammatory bowel disease (IBD), the amount of GLP-2 was significantly reduced in the colon[86], however, others report that TNBS-induced ileitis results in an increase in colonic GLP-2 immunoreactivity. (O'Hara and Sharkey, personal communications). Regarding the latter, perhaps an increase in local and circulatory GLP-2 is the result of IBD-induced diarrhea, thus increasing the nutrient load in the distal bowel, a well-described potent stimulus for inducing GLP-2 production[12,15,17]. Though speculative, perhaps increased growth and nutrient absorption in the proximal small intestine as a result of increased GLP-2 production would be successful in ameliorating inflammatory processes. Conversely, Schmidt *et al* found that both the fasting and meal-induced plasma levels of GLP-2 were not different between healthy controls and IBD patients[87]. This report suggests that L-cell secretion of GLP-2 is not altered in IBD. The discrepancies between these observations could be the result of model/species difference, or site-specific effects of inflammation in the bowel.

While GLP-2 is the most prominent enterotrophic peptide, there are a number of hormones that might be involved in the regulation of mucosal growth. These are briefly discussed as it is likely that processes involved in intestinal adaptation are regulated by a complex array of hormones, neuropeptides, and cytokines. A number of mucosal growth factors that might be useful for the treatment of short bowel syndrome are listed in Table 1.

Insulin-like growth factor-I

Another growth factor that might have a potential role in enhancing mucosal growth is insulin-like growth factor-I (IGF-I). IGF-I is produced mainly in the liver, but it is also synthesized locally in the gut[88]. There is evidence that IGF-I stimulates intestinal growth under experimental conditions of TPN therapy, intestinal resection, and radiation therapy[89]. In addition, the over-expression of the gene encoding for IGF-I is associated with increased small bowel length, small bowel weight, and crypt cell proliferation[90]. Conversely, others report that ileal IGF-I levels remain unchanged following intestinal resection[90,91].

Most of the circulating IGF-I is bound to IGF-binding proteins (IGFBPs) that potentially protect IGF-I from degradation thereby modulating the activity of IGF-I[92,93]. IGFBPs are capable of controlling the availability of circulating IGF-I to target tissues and are thus key components in the GH-IGF-I somatotropic axis. In humans, six IGFBPs have been identified and shown to modulate IGF-I actions differently[94]. Serum levels, jejunal tissue protein, and mRNA levels of IGF-I are reduced following small bowel resection[95]. One possibility is that there is a significant decrease in IGFBP-3 mRNA following intestinal resection, thus this rapid decrease in the IGFBP-3 could increase IGF-I bioavailability resulting in an enhanced adaptive response[96].

In SBS rats maintained on parenteral nutrition, IGF-I infusions induced jejunal hyperplasia and normalized ion transport[97]. Thus acute IGF-I treatments might prove to be beneficial in easing the transition from parental to enteral feeds thereby avoiding some of the long-term dilemmas associated with TPN therapy[98]. However, there is no significant evidence of serum or tissue concentration fluctuation of IGF-I following intestinal resection, therefore its utility as a treatment for SBS remains unclear.

Epidermal growth factor

Most of the EGF family of peptides (transforming growth factor-α, amphiregulin, heparin-binding EGF, epiregulin, betacellulin, neuregulin, neuregulin-2) are trophic to the gastrointestinal tract as they both stimulate crypt cell proliferation and suppress apoptosis[99]. There is evidence that EGF administration at the time of small bowel resection enhances the adaptive response. This appears to be accomplished by inducing mucosal hyperplasia and increasing nutrient absorption, while reducing intestinal permeability, weight loss, and apoptosis[49,100,101]. It is important to note that EGF therapy is most effective for intestinal adaptation when it is administered early following resection[102]. Very few studies have examined serum EGF levels following small bowel resection. Shin *et al* showed that small bowel resection does not change serum EGF levels, yet there was an increase in salivary and a decrease in urinary excretion of EGF[103]. Ileal epidermal growth factor receptor (EGFR) levels were significantly increased suggesting that the increased salivary EGF (significant endogenous source of EGF), enhanced EGF-R expression, and reduced urinary excretion of EGF, leads to an increase in ileal utilization of EGF following resection. Compared with control mice, the removal of the submandibular glands significantly attenuated the increase in villus height, total protein, and DNA content of the small bowel following massive small bowel resection[104].

It has also been suggested that intestinal adaptation following resection requires a functional EGFR[105]. Selective inhibition of the EGFR with an orogastric EGFR inhibitor (ZD1839, 50 mg/kg per day) results in impaired intestinal adaptation after small bowel resection[106,107]. There is also an impaired adaptive response to intestinal resection in waved-2 mice-mice that are predisposed to a naturally occurring mutation that results in a reduction in EGFR protein tyrosine kinase activity. *In vitro* studies demonstrate that serum from intestinal resection mice or rats is capable of stimulating a proliferative response in rat intestinal epithelial cells (IEC)[108]. This uncharacterized factor released into the serum of 7 d resected rats significantly increased EGF-R protein expression. Additionally, the proliferative response in IEC's was abolished following the addition of a specific EGFR inhibitor. It would be intriguing to explore if there is cross talk between GLP-2, which is significantly increased following intestinal resection, and the EGFR signaling pathway. The basis for this speculation was

discussed earlier and is depicted in Figure 3B.

PACAP and VIP

All but one of the members of the PACAP/glucagon superfamily, GIP, have been found as protein and/or mRNA in the brain and are thus classified as neuropeptides[34]. For an excellent review of this family of peptides, see Sherwood *et al*[34]. All of these peptides are found in the gut and signal through 7-TM G-protein coupled receptors. In humans, the present members include PACAP, glucagon, glucagon-like peptide-1, growth hormone releasing hormone, vasoactive intestinal peptide (VIP), secretin, glucose-dependent insulinotropic peptide (GIP), and GLP-2[34]. Both VIP and PACAP have a widespread distribution and are known to affect neural, circulatory, gastrointestinal, endocrine, and immune systems. There is evidence that VIP and PACAP administration reduces apoptosis and promotes the survival of neural cells by inducing *bcl-2* activity. Increased *bcl-2* activity is associated with an inhibition of caspase-3 activity and decreased cytoplasmic cytochrome C release[34]. Moreover, the BH4 domain of the pro-survival protein *bcl-2* both binds to the C-terminus of Apaf-1 on caspase-9, thus inhibiting caspase-induced apoptosis, as well as directly or indirectly preventing the release of cytochrome C from the mitochondria[109,110]. Similar results were attained following the activation of GLP-2R signaling by GLP-2 administration in transfected cells treated with cyclohexamide or irinotecan. These data show that there was an inhibition of apoptosis, reduced caspase activation, and decreased mitochondrial cytochrome C release[57,84]. An additional parallel of these peptides is revealed following recent studies suggesting that GLP-2 is a vasodilator[111,112]. PACAP and VIP are both established as potent vasodilators[34].

At physiological concentrations, VIP and GLP-2 are both ineffective in stimulating cell growth directly in cell culture. Perhaps the intact ENS might need to be activated first as VIP receptors, and likely GLP-2 receptors, are located on enteric neurons[34,51]. It would be interesting to examine the growth-promoting potential of the ENS on epithelial cells *in vivo*.

There are conflicting results regarding plasma and tissue levels of VIP following small bowel resection and in studies examining SBS. These differences may be species-specific as following intestinal resection, dogs and pigs have a significant reduction in VIP levels, whereas in humans, there is either an elevation or no change in plasma VIP levels[113-116]. There is no evidence that VIP and PACAP administration is directly trophic for the small bowel thus a role for VIP in small bowel resection in the rat has not been described.

Peptide YY and Cholecystokinin

Further study is required when looking at the relationship of other co-localized mediators that are released from the L-cell. Peptide tyrosine tyrosine (PYY) is a 36 amino acid gastrointestinal peptide present in endocrine L-cells of the ileal, colonic, and rectal mucosa[117-121]. PYY is co-localized to some degree with GLP-1/GLP-2 in the L-cells[122,123], moreover, it generates many of the physiological actions that are also attributed to GLP-2. There are data that both GLP-2 and PYY: slow gastric emptying and intestinal transit; production rates in rats and humans are increased following intestinal resection, production rates are influenced by luminal nutrients in the hindgut; release might be influenced by a proximal gut hormone or perhaps a neuroendocrine mechanism[51,124-133]. In support of the latter, our results examining postprandial GLP-2 release[15], together with reports of the meal-stimulated PYY response[133], demonstrate that there are significant increases in the serum levels of both peptides in less than 15 min. Intestinal transit times are not that rapid, thus these early increases probably are not attributable to luminal nutrients in the distal intestine. Neuroendocrine factor induction of the early release of PYY was described in a study by Greeley *et al*[121]. They administered a meal to dogs and then diverted the entire meal out of the proximal intestine via the creation of a stoma. Thus, there was no possibility of nutrient stimulation of PYY containing L-cells in the distal gut. Interestingly, there was still significant increase in plasma PYY levels. In these same dogs, the removal of the ileum, colon, and rectum, resulted in no increase in plasma PYY levels in response to a meal. These findings replicate a human study previously discussed in this paper, but instead of PYY, GLP-2 levels in patients that received a colectomy were significantly reduced[47]. This is certainly supportive of either a blood-borne hormone or a neural pathway in the generation of the early release of PYY and most assuredly, GLP-2. Furthermore, we have observed that rats with 20 cm of jejunum diverted out an abdominal stoma (i.e. no possibility of meal contents directly contacting the distal bowel) still had a significant increase in post-prandial GLP-2 levels-unpublished data-(Figure 5).

Several proximal gut hormones that are rapidly secreted following the ingestion of a meal have been tested (i.e., gastrin, glucose dependent insulinotropic peptide, secretin, neurotensin) and found to be ineffective in stimulating an increase in PYY[121]. Currently, no published literature has shown that these can stimulate GLP-2 release. One potential agonist, cholecystokinin (CCK), induces a dose-dependent release of PYY, and this CCK-induced PYY release, can be blocked by a CCK antagonist[121]. As CCK induces PYY production from L-cells, and having established that PYY and GLP-2 are co-localized, potentially, CCK might also be a foregut hormone responsible for stimulating the early release of GLP-2. It would be worthwhile to examine whether CCK also triggers GLP-2 secretion.

PYY administration has not shown that it is trophic to the gastrointestinal tract directly and it is unlikely that it is involved in the adaptive response following intestinal resection, though a reduction in intestinal transit may secondarily generate increased intestinal growth. These areas have not been explored. Both GLP-2 and PYY are inactivated by dipeptyl peptidases[75,76], thus additional studies of SBS should explore the relationship between serum and intestinal tissue levels of GLP-2, PYY, and DPP-IV.

Polyamines

Polyamines are present in prokaryotes, plants and animals. The polyamines putrescine and its derivatives spermidine and spermine are found in nearly all cells of higher eukaryotes[134,135]. The major role of polyamines in most cell types is to stimulate cell proliferation and thus is considered essential for life[135,136]. The inhibition of polyamine biosynthesis blocks cell growth[136,137]. The increase in intracellular polyamine levels and in the activity of ornithine decarboxylase (ODC), one of the rate-limiting enzymes in the strictly controlled polyamine biosynthetic pathway[138], are associated with rapid growth rates[139,140]. ODC activity in the rat small intestinal mucosa is increased following small bowel resection[134,138], parasitic or enteropathogenic bacteria-induced small intestinal inflammation or colitis[141,142], ischemia reperfusion[143], and following partial obstruction of the lumen. All of these instances are associated with an increase in mucosal growth. Of clinical relevance, mucosal ODC activity in the colon and rectum has been reported to be significantly higher in both ulcerative colitis and Crohn's disease patients[144]. In addition, evidence shows that polyamines are protective of DNA during the S-phase of the growth cycle[135,145] and in part may explain the protective effects of GLP-2 following the administration of chemotherapeutic agents.

There is a rise in plasma enteroglucagon levels that precedes the activation of ODC[143]. Thus it could be speculated that an increase in GLP-2 might initiate the activation of ODC leading to the repair of injured intestinal mucosa. If so, it would not be unreasonable for the growth promoting and protective role of GLP-2 in the small bowel to act through an ODC-dependent formation of polyamines. Considering that the trophic effects of many growth-promoting gut hormones are blocked following ODC or polyamine synthesis inhibition[134,138], it would not be surprising if this pathway is involved as a downstream effector of GLP-2 induced intestinal growth.

Intestinal carcinogenesis

A great deal of attention has focused on the potential for cancerous growth attributable as a side effect following the application of growth factors. Recent results cautioning restraint regarding the utility of GLP-2 was indicated as GLP-2, and to a greater degree, a GLP-2 analog, promoted an increase in the growth of mucosal neoplasms in mice pre-induced to form colonic tumours[146]. Furthermore, there was a significant increase in tumour load in the mice treated with either GLP-2, or the synthetic Gly2-GLP-2 analogue. There is also evidence that GLP-2R mRNA transcripts can be detected in two human cervical carcinoma cell lines[56]. But does the application of growth factors such as GLP-2 or EGF always trigger neoplastic growth? Could the application of growth factors prevent the development of intestinal neoplasms? Perhaps the primary factor involved in neoplastic growth is not necessarily the addition of the growth factor, but rather a dysfunction in downstream receptor (i.e., mutation) or signal transduction pathways. For example, there is evidence that mutations in cytoplasmic signaling elements involved in the EGFR signaling cascade result in an alteration in signal transduction events that lead to changes in the downregulation/internalization of the EGFR[147]. Perhaps what occurs in established oncogenic cells is that a mutation in an element of the signaling cascade prevents GLP-2- or EGF-induced receptor downregulation. This theory is supported by recent work that shows that an oncogenic form of c-Cbl/c-Cbl, which stabilizes and ubiquitinates the cytoplasmic region of the EGFR, is able to prevent EGF-induced EGFR down regulation thus bypassing degradative pathways[148]. The consequences of this mutation are a bypass of receptor internalization and desensitization processes, an induction in the over expression of the EGFR, and finally, aberrant cell growth. A downstream defect in trafficking could potentially be the primary cause of neoplastic, abnormal cell growth, not necessarily the application of the growth factor.

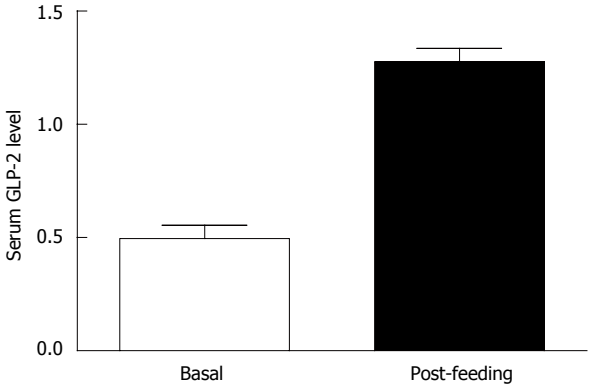

Figure 5 Neural or humoral mechanism involvement in the early release of GLP-2. The delivery of a meal significantly increases serum GLP-2 levels in rats with discontinuous small bowel (ANOVA, $P < 0.007$). The disruption of the continuity of the bowel (stoma creation) did not inhibit the initial nutrient-stimulated GLP-2 response. Thus, GLP-2 production is increased at least in part by neural and/or hormonal mechanisms. Serum GLP-2 levels reported as ng/mL.

In the case of the promotion of tumour size in mice following GLP-2 treatment as noted by Thulesen *et al*, or potentially as in the CaCO-2, T84, HeLa cells[56,58,59], what has occurred is that dysfunctional receptors and/or signal transduction pathways are already present within/on these cell types. As with any neoplastic condition, the application of growth factors is always a risk. Potentially the application of growth factors prior to the development of gastrointestinal neoplasms, such as during the early stages of Crohn's disease or ulcerative colitis, would be beneficial in halting the progress of prolonged inflammation. It is well established that prolonged intestinal inflammation often secondarily leads to the development of mutations, cell dysfunction, and carcinogenic growth.

CONCLUSIONS

The trophic effect of GLP-2 on the intestine demonstrates that it is possible to modify or accelerate the process of intestinal adaptation, which would potentially make GLP-2 useful for the treatment of short bowel syndrome. Evidence shows that increases in GLP-2 production following intestinal resection are significantly correlated with intestinal adaptation. The association between an increase in nutrient malabsorption and GLP-2 production

following intestinal resection has led to speculations that GLP-2 may act to control the nutrient absorptive capacity within the bowel. The increased availability of circulatory GLP-2 might then stimulate proximal mucosal growth resulting in enhanced nutrient absorptive capacity in the remaining intestinal remnant. Moreover, GLP-2 administration maintains epithelial barrier function and increases both crypt cell proliferation and weight gain in the absence of enteral nutrients. This supports the further development of GLP-2 as a therapeutic strategy that could enhance intestinal adaptation and reduce the consequences of parenteral feeding.

Although significant progress has been made towards elucidating the mechanisms mediating the trophic effects of GLP-2, several formidable challenges lie ahead. The biological and signaling role of the GLP-2R has not been fully defined, thus the mechanistic pathway (s) by which GLP-2 induces adaptive change in the small bowel presently remain elusive. Data showing that GLP-2 administration significantly increases cell proliferation in the absence of the characterized GLP-2R strongly suggest that GLP-2's trophic effect might also include activation of either an uncharacterized GLP-2R or the transactivation of other receptor/signaling paradigms. Another key goal should be in determining the mechanism involved in the early release of GLP-2 following a meal or in malabsorptive states. Lastly, an emergent area of interest is the potential of GLP-2 to induce stimulation of the ENS as an intermediate in the regulation of intestinal growth. Regardless, there is a very significant and biologically important effect of GLP-2 on intestinal function and whole animal physiology that strongly supports further research into the use of GLP-2 as a therapy. The future use of GLP-2 therapy for the treatment of intestinal disorders is an enticing prospect; however, the promise of these initial results must be tempered as we await confirmatory data from clinical trials.

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REFERENCES

1 **Dowling RH**, Booth CC. Structural and functional changes following small intestinal resection in the rat. *Clin Sci* 1967; **32**: 139-149
2 **Vanderhoof JA**. Short bowel syndrome in children and small intestinal transplantation. *Pediatr Clin North Am* 1996; **43**: 533-550
3 **Williamson RC**. Intestinal adaptation (first of two parts). Structural, functional and cytokinetic changes. *N Engl J Med* 1978; **298**: 1393-1402
4 **Sigalet DL**. Short bowel syndrome in infants and children: an overview. *Semin Pediatr Surg* 2001; **10**: 49-55
5 **Vanderhoof JA**, Langnas AN, Pinch LW, Thompson JS, Kaufman SS. Short bowel syndrome. *J Pediatr Gastroenterol Nutr* 1992; **14**: 359-370
6 **Vanderhoof JA**, Langnas AN. Short-bowel syndrome in children and adults. *Gastroenterology* 1997; **113**: 1767-1778
7 **Meehan JJ**, Georgeson KE. Prevention of liver failure in parenteral nutrition-dependent children with short bowel syndrome. *J Pediatr Surg* 1997; **32**: 473-475
8 **Briones ER**, Iber FL. Liver and biliary tract changes and injury associated with total parenteral nutrition: pathogenesis and prevention. *J Am Coll Nutr* 1995; **14**: 219-228
9 **Baksheev L**, Fuller PJ. Humoral factors in intestinal adaptation. *Trends Endocrinol Metab* 2000; **11**: 401-405
10 **Podolsky DK**. Regulation of intestinal epithelial proliferation: a few answers, many questions. *Am J Physiol* 1993; **264**: G179-G186
11 **Lovshin J**, Drucker DJ. New frontiers in the biology of GLP-2. *Regul Pept* 2000; **90**: 27-32
12 **Xiao Q**, Boushey RP, Drucker DJ, Brubaker PL. Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. *Gastroenterology* 1999; **117**: 99-105
13 **Brubaker PL**, Crivici A, Izzo A, Ehrlich P, Tsai CH, Drucker DJ. Circulating and tissue forms of the intestinal growth factor, glucagon-like peptide-2. *Endocrinology* 1997; **138**: 4837-4843
14 **Hartmann B**, Harr MB, Jeppesen PB, Wojdemann M, Deacon CF, Mortensen PB, Holst JJ. In vivo and in vitro degradation of glucagon-like peptide-2 in humans. *J Clin Endocrinol Metab* 2000; **85**: 2884-2888
15 **Martin GR**, Wallace LE, Hartmann B, Holst JJ, Demchyshyn L, Toney K, Sigalet DL. Nutrient-stimulated GLP-2 release and crypt cell proliferation in experimental short bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G431-G438
16 **van Goudoever JB**, Stoll B, Hartmann B, Holst JJ, Reeds PJ, Burrin DG. Secretion of trophic gut peptides is not different in bolus- and continuously fed piglets. *J Nutr* 2001; **131**: 729-732
17 **Burrin DG**, Stoll B. Key nutrients and growth factors for the neonatal gastrointestinal tract. *Clin Perinatol* 2002; **29**: 65-96
18 **Martin GR**, Wallace LE, Sigalet DL. Glucagon-like peptide-2 induces intestinal adaptation in parenterally fed rats with short bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G964-G972
19 **Burrin DG**, Stoll B, Jiang R, Petersen Y, Elnif J, Buddington RK, Schmidt M, Holst JJ, Hartmann B, Sangild PT. GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G1249-G1256
20 **Burrin DG**, Stoll B, Guan X, Cui L, Chang X, Holst JJ. Glucagon-like peptide 2 dose-dependently activates intestinal cell survival and proliferation in neonatal piglets. *Endocrinology* 2005; **146**: 22-32
21 **Sigalet DL**, Martin GR. Hormonal therapy for short bowel syndrome. *J Pediatr Surg* 2000; **35**: 360-363; discussion 364
22 **Scott RB**, Kirk D, MacNaughton WK, Meddings JB. GLP-2 augments the adaptive response to massive intestinal resection in rat. *Am J Physiol* 1998; **275**: G911-G921
23 **Warner BW**. GLP-2 as therapy for the short-bowel syndrome. *Gastroenterology* 2001; **120**: 1041-1043
24 **Drucker DJ**. Biological actions and therapeutic potential of the glucagon-like peptides. *Gastroenterology* 2002; **122**: 531-544
25 **Tsai CH**, Hill M, Asa SL, Brubaker PL, Drucker DJ. Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. *Am J Physiol* 1997; **273**: E77-E84
26 **Jeppesen PB**, Sanguinetti EL, Buchman A, Howard L, Scolapio JS, Ziegler TR, Gregory J, Tappenden KA, Holst J, Mortensen PB. Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients. *Gut* 2005; **54**: 1224-1231
27 **Bloom SR**. An enteroglucagon tumour. *Gut* 1972; **13**: 520-523
28 **Gleeson MH**, Bloom SR, Polak JM, Henry K, Dowling RH. Endocrine tumour in kidney affecting small bowel structure, motility, and absorptive function. *Gut* 1971; **12**: 773-782
29 **Drucker DJ**, Erlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc Natl Acad Sci USA* 1996; **93**: 7911-7916
30 **Brubaker PL**, Izzo A, Hill M, Drucker DJ. Intestinal function in mice with small bowel growth induced by glucagon-like

peptide-2. *Am J Physiol* 1997; **272**: E1050-E1058
31 **Drucker DJ**, DeForest L, Brubaker PL. Intestinal response to growth factors administered alone or in combination with human [Gly2]glucagon-like peptide 2. *Am J Physiol* 1997; **273**: G1252-G1262
32 **Chance WT**, Foley-Nelson T, Thomas I, Balasubramaniam A. Prevention of parenteral nutrition-induced gut hypoplasia by coinfusion of glucagon-like peptide-2. *Am J Physiol* 1997; **273**: G559-G563
33 **Hartmann B**, Thulesen J, Hare KJ, Kissow H, Orskov C, Poulsen SS, Holst JJ. Immunoneutralization of endogenous glucagon-like peptide-2 reduces adaptive intestinal growth in diabetic rats. *Regul Pept* 2002; **105**: 173-179
34 **Sherwood NM**, Krueckl SL, McRory JE. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* 2000; **21**: 619-670
35 **Alavi K**, Schwartz MZ, Palazzo JP, Prasad R. Treatment of inflammatory bowel disease in a rodent model with the intestinal growth factor glucagon-like peptide-2. *J Pediatr Surg* 2000; **35**: 847-851
36 **Bell GI**, Sanchez-Pescador R, Laybourn PJ, Najarian RC. Exon duplication and divergence in the human preproglucagon gene. *Nature* 1983; **304**: 368-371
37 **Bell GI**, Santerre RF, Mullenbach GT. Hamster preproglucagon contains the sequence of glucagon and two related peptides. *Nature* 1983; **302**: 716-718
38 **Orskov C**, Holst JJ, Knuhtsen S, Baldissera FG, Poulsen SS, Nielsen OV. Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from pig small intestine but not pancreas. *Endocrinology* 1986; **119**: 1467-1475
39 **Furuta M**, Yano H, Zhou A, Rouillé Y, Holst JJ, Carroll R, Ravazzola M, Orci L, Furuta H, Steiner DF. Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *Proc Natl Acad Sci USA* 1997; **94**: 6646-6651
40 **Holst JJ**. Gut hormones as pharmaceuticals. From enteroglucagon to GLP-1 and GLP-2. *Regul Pept* 2000; **93**: 45-51
41 **Damholt AB**, Buchan AM, Holst JJ, Kofod H. Proglucagon processing profile in canine L cells expressing endogenous prohormone convertase 1/3 and prohormone convertase 2. *Endocrinology* 1999; **140**: 4800-4808
42 **Dey A**, Lipkind GM, Rouillé Y, Norrbom C, Stein J, Zhang C, Carroll R, Steiner DF. Significance of prohormone convertase 2, PC2, mediated initial cleavage at the proglucagon interdomain site, Lys70-Arg71, to generate glucagon. *Endocrinology* 2005; **146**: 713-727
43 **Webb GC**, Dey A, Wang J, Stein J, Milewski M, Steiner DF. Altered proglucagon processing in an alpha-cell line derived from prohormone convertase 2 null mouse islets. *J Biol Chem* 2004; **279**: 31068-31075
44 **Irwin DM**, Wong J. Trout and chicken proglucagon: alternative splicing generates mRNA transcripts encoding glucagon-like peptide 2. *Mol Endocrinol* 1995; **9**: 267-277
45 **Irwin DM**. Molecular evolution of proglucagon. *Regul Pept* 2001; **98**: 1-12
46 **Jeppesen PB**, Hartmann B, Thulesen J, Hansen BS, Holst JJ, Poulsen SS, Mortensen PB. Elevated plasma glucagon-like peptide 1 and 2 concentrations in ileum resected short bowel patients with a preserved colon. *Gut* 2000; **47**: 370-376
47 **Jeppesen PB**, Hartmann B, Hansen BS, Thulesen J, Holst JJ, Mortensen PB. Impaired meal stimulated glucagon-like peptide 2 response in ileal resected short bowel patients with intestinal failure. *Gut* 1999; **45**: 559-563
48 **Caddy G**, Johnston C, Ardill J, Pogue K, Collins J, Gardiner K, Watson P. Distribution of glucagon-like peptide-2 in normal colonic tissue. *Scand J Gastroenterol* 2003; **38**: 798-799
49 **Sham J**, Martin G, Meddings JB, Sigalet DL. Epidermal growth factor improves nutritional outcome in a rat model of short bowel syndrome. *J Pediatr Surg* 2002; **37**: 765-769
50 **Yusta B**, Huang L, Munroe D, Wolff G, Fantaske R, Sharma S, Demchyshyn L, Asa SL, Drucker DJ. Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. *Gastroenterology* 2000; **119**: 744-755
51 **Bjerknes M**, Cheng H. Modulation of specific intestinal epithelial progenitors by enteric neurons. *Proc Natl Acad Sci USA* 2001; **98**: 12497-12502
52 **Ørskov C**, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. *Regul Pept* 2005; **124**: 105-112
53 **Munroe DG**, Gupta AK, Kooshesh F, Vyas TB, Rizkalla G, Wang H, Demchyshyn L, Yang ZJ, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, Crivici A. Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proc Natl Acad Sci USA* 1999; **96**: 1569-1573
54 **Lovshin J**, Estall J, Yusta B, Brown TJ, Drucker DJ. Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling. *J Biol Chem* 2001; **276**: 21489-21499
55 **Yusta B**, Somwar R, Wang F, Munroe D, Grinstein S, Klip A, Drucker DJ. Identification of glucagon-like peptide-2 (GLP-2)-activated signaling pathways in baby hamster kidney fibroblasts expressing the rat GLP-2 receptor. *J Biol Chem* 1999; **274**: 30459-30467
56 **Koehler JA**, Yusta B, Drucker DJ. The HeLa cell glucagon-like peptide-2 receptor is coupled to regulation of apoptosis and ERK1/2 activation through divergent signaling pathways. *Mol Endocrinol* 2005; **19**: 459-473
57 **Boushey RP**, Yusta B, Drucker DJ. Glucagon-like peptide (GLP)-2 reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor. *Cancer Res* 2001; **61**: 687-693
58 **Rocha FG**, Shen KR, Jasleen J, Tavakkolizadeh A, Zinner MJ, Whang EE, Ashley SW. Glucagon-like peptide-2: divergent signaling pathways. *J Surg Res* 2004; **121**: 5-12
59 **Jasleen J**, Ashley SW, Shimoda N, Zinner MJ, Whang EE. Glucagon-like peptide 2 stimulates intestinal epithelial proliferation in vitro. *Dig Dis Sci* 2002; **47**: 1135-1140
60 **Bulut K**, Meier JJ, Ansorge N, Felderbauer P, Schmitz F, Hoffmann P, Schmidt WE, Gallwitz B. Glucagon-like peptide 2 improves intestinal wound healing through induction of epithelial cell migration in vitro-evidence for a TGF--beta-mediated effect. *Regul Pept* 2004; **121**: 137-143
61 **Kim KA**, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, Funk WD, Tomizuka K. Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* 2005; **309**: 1256-1259
62 **Batlle E**, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 2002; **111**: 251-263
63 **Potten CS**, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 1990; **110**: 1001-1020
64 **Potten CS**, Owen G, Roberts SA. The temporal and spatial changes in cell proliferation within the irradiated crypts of the murine small intestine. *Int J Radiat Biol* 1990; **57**: 185-199
65 **Velázquez E**, Ruiz-Albusac JM, Blázquez E. Glucagon-like peptide-2 stimulates the proliferation of cultured rat astrocytes. *Eur J Biochem* 2003; **270**: 3001-3009
66 **Tang-Christensen M**, Larsen PJ, Thulesen J, Rømer J, Vrang N. The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. *Nat Med* 2000; **6**: 802-807
67 **Sayegh AI**, Ritter RC. Vagus nerve participates in CCK-induced Fos expression in hindbrain but not myenteric plexus. *Brain Res* 2000; **878**: 155-162
68 **Sayegh AI**, Ritter RC. CCK-A receptor activation induces fos expression in myenteric neurons of rat small intestine. *Regul Pept* 2000; **88**: 75-81
69 **Larsen PJ,** Vrang N, Orskov C, Hartmann B, Holst JJ, Tang-Christensen M. The central glucagon-like peptide-2 (GLP-2) containing pathway is involved in regulation of food intake in rats. *Obesity Research* 2001; **9**: 55S

70 **Tang-Christensen M,** Vrang N, Hartmann B, Larsen LK, Orskov C, Holst JJ, Larsen PJ. The correlation between feeding status and the amount of proglucagon, GLP-1 and GLP-2 in the central nervous system of the rat. *Diabetes* 2001; **50**: A373

71 **Sørensen LB**, Flint A, Raben A, Hartmann B, Holst JJ, Astrup A. No effect of physiological concentrations of glucagon-like peptide-2 on appetite and energy intake in normal weight subjects. *Int J Obes Relat Metab Disord* 2003; **27**: 450-456

72 **Thulesen J**, Knudsen LB, Hartmann B, Hastrup S, Kissow H, Jeppesen PB, Ørskov C, Holst JJ, Poulsen SS. The truncated metabolite GLP-2 (3-33) interacts with the GLP-2 receptor as a partial agonist. *Regul Pept* 2002; **103**: 9-15

73 **Shin ED**, Estall JL, Izzo A, Drucker DJ, Brubaker PL. Mucosal adaptation to enteral nutrients is dependent on the physiologic actions of glucagon-like peptide-2 in mice. *Gastroenterology* 2005; **128**: 1340-1353

74 **Drucker DJ**, Shi Q, Crivici A, Sumner-Smith M, Tavares W, Hill M, DeForest L, Cooper S, Brubaker PL. Regulation of the biological activity of glucagon-like peptide 2 in vivo by dipeptidyl peptidase IV. *Nat Biotechnol* 1997; **15**: 673-677

75 **Mentlein R**. Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. *Regul Pept* 1999; **85**: 9-24

76 **Lambeir AM**, Durinx C, Scharpé S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Crit Rev Clin Lab Sci* 2003; **40**: 209-294

77 **Drucker DJ**, Boushey RP, Wang F, Hill ME, Brubaker PL, Yusta B. Biologic properties and therapeutic potential of glucagon-like peptide-2. *JPEN J Parenter Enteral Nutr* 1999; **23**: S98-100

78 **Geier MS**, Tenikoff D, Yazbeck R, McCaughan GW, Abbott CA, Howarth GS. Development and resolution of experimental colitis in mice with targeted deletion of dipeptidyl peptidase IV. *J Cell Physiol* 2005; **204**: 687-692

79 **Orskov C**, Andreasen J, Holst JJ. All products of proglucagon are elevated in plasma from uremic patients. *J Clin Endocrinol Metab* 1992; **74**: 379-384

80 **Ruiz-Grande C**, Pintado J, Alarcón C, Castilla C, Valverde I, López-Novoa JM. Renal catabolism of human glucagon-like peptides 1 and 2. *Can J Physiol Pharmacol* 1990; **68**: 1568-1573

81 **Tavares W**, Drucker DJ, Brubaker PL. Enzymatic- and renal-dependent catabolism of the intestinotropic hormone glucagon-like peptide-2 in rats. *Am J Physiol Endocrinol Metab* 2000; **278**: E134-E139

82 **Boushey RP**, Yusta B, Drucker DJ. Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. *Am J Physiol* 1999; **277**: E937-E947

83 **Drucker DJ**, Yusta B, Boushey RP, DeForest L, Brubaker PL. Human [Gly2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. *Am J Physiol* 1999; **276**: G79-G91

84 **Tavakkolizadeh A**, Shen R, Abraham P, Kormi N, Seifert P, Edelman ER, Jacobs DO, Zinner MJ, Ashley SW, Whang EE. Glucagon-like peptide 2: a new treatment for chemotherapy-induced enteritis. *J Surg Res* 2000; **91**: 77-82

85 **Booth C**, Booth D, Williamson S, Demchyshyn LL, Potten CS. Teduglutide ([Gly2]GLP-2) protects small intestinal stem cells from radiation damage. *Cell Prolif* 2004; **37**: 385-400

86 **Schmidt PT**, Hartmann B, Bregenholt S, Hoist JJ, Claesson MH. Deficiency of the intestinal growth factor, glucagon-like peptide 2, in the colon of SCID mice with inflammatory bowel disease induced by transplantation of CD4+ T cells. *Scand J Gastroenterol* 2000; **35**: 522-527

87 **Schmidt PT**, Ljung T, Hartmann B, Hare KJ, Holst JJ, Hellström PM. Tissue levels and post-prandial secretion of the intestinal growth factor, glucagon-like peptide-2, in controls and inflammatory bowel disease: comparison with peptide YY. *Eur J Gastroenterol Hepatol* 2005; **17**: 207-212

88 **Chen K**, Nezu R, Wasa M, Sando K, Kamata S, Takagi Y, Okada A. Insulin-like growth factor-1 modulation of intestinal epithelial cell restitution. *JPEN J Parenter Enteral Nutr* 1999; **23**: S89-S92

89 **Lund PK**. Molecular basis of intestinal adaptation: the role of the insulin-like growth factor system. *Ann N Y Acad Sci* 1998; **859**: 18-36

90 **Wang J**, Niu W, Nikiforov Y, Naito S, Chernausek S, Witte D, LeRoith D, Strauch A, Fagin JA. Targeted overexpression of IGF-I evokes distinct patterns of organ remodeling in smooth muscle cell tissue beds of transgenic mice. *J Clin Invest* 1997; **100**: 1425-1439

91 **Vanderhoof JA**, McCusker RH, Clark R, Mohammadpour H, Blackwood DJ, Harty RF, Park JH. Truncated and native insulinlike growth factor I enhance mucosal adaptation after jejunoileal resection. *Gastroenterology* 1992; **102**: 1949-1956

92 **Lund PK**. Insulin-like growth factor I: molecular biology and relevance to tissue-specific expression and action. *Recent Prog Horm Res* 1994; **49**: 125-148

93 **Ney DM**, Huss DJ, Gillingham MB, Kritsch KR, Dahly EM, Talamantez JL, Adamo ML. Investigation of insulin-like growth factor (IGF)-I and insulin receptor binding and expression in jejunum of parenterally fed rats treated with IGF-I or growth hormone. *Endocrinology* 1999; **140**: 4850-4860

94 **Rosenzweig SA**. What's new in the IGF-binding proteins? *Growth Horm IGF Res* 2004; **14**: 329-336

95 **Albiston AL**, Herington AC. Tissue distribution and regulation of insulin-like growth factor (IGF)-binding protein-3 messenger ribonucleic acid (mRNA) in the rat: comparison with IGF-I mRNA expression. *Endocrinology* 1992; **130**: 497-502

96 **Albiston AL**, Taylor RG, Herington AC, Beveridge DJ, Fuller PJ. Divergent ileal IGF-I and IGFBP-3 gene expression after small bowel resection: a novel mechanism to amplify IGF action? *Mol Cell Endocrinol* 1992; **83**: R17-R20

97 **Ney DM**. Effects of insulin-like growth factor-I and growth hormone in models of parenteral nutrition. *JPEN J Parenter Enteral Nutr* 1999; **23**: S184-S189

98 **Gillingham MB**, Dahly EM, Murali SG, Ney DM. IGF-I treatment facilitates transition from parenteral to enteral nutrition in rats with short bowel syndrome. *Am J Physiol Regul Integr Comp Physiol* 2003; **284**: R363-R371

99 **Barnard JA**, Beauchamp RD, Russell WE, Dubois RN, Coffey RJ. Epidermal growth factor-related peptides and their relevance to gastrointestinal pathophysiology. *Gastroenterology* 1995; **108**: 564-580

100 **Thompson JS**. Epidermal growth factor and the short bowel syndrome. *JPEN J Parenter Enteral Nutr* 1999; **23**: S113-S116

101 **Helmrath MA**, Shin CE, Erwin CR, Warner BW. The EGF\ EGF-receptor axis modulates enterocyte apoptosis during intestinal adaptation. *J Surg Res* 1998; **77**: 17-22

102 **Shin CE**, Helmrath MA, Falcone RA Jr, Fox JW, Duane KR, Erwin CR, Warner BW. Epidermal growth factor augments adaptation following small bowel resection: optimal dosage, route, and timing of administration. *J Surg Res* 1998; **77**: 11-16

103 **Shin CE**, Falcone RA Jr, Duane KR, Erwin CR, Warner BW. The distribution of endogenous epidermal growth factor after small bowel resection suggests increased intestinal utilization during adaptation. *J Pediatr Surg* 1999; **34**: 22-26

104 **Helmrath MA**, Shin CE, Fox JW, Erwin CR, Warner BW. Adaptation after small bowel resection is attenuated by sialoadenectomy: the role for endogenous epidermal growth factor. *Surgery* 1998; **124**: 848-854

105 **Stern LE**, Erwin CR, O'Brien DP, Huang FS, Warner BW. Serum from mice after small bowel resection enhances intestinal epithelial cell growth. *J Pediatr Surg* 2001; **36**: 184-189

106 **O'Brien DP**, Nelson LA, Williams JL, Kemp CJ, Erwin CR, Warner BW. Selective inhibition of the epidermal growth factor receptor impairs intestinal adaptation after small bowel resection. *J Surg Res* 2002; **105**: 25-30

107 **Helmrath MA**, Erwin CR, Warner BW. A defective EGF-receptor in waved-2 mice attenuates intestinal adaptation. *J Surg Res* 1997; **69**: 76-80

108 **Juno RJ**, Williams JL, Knott AW, Erwin CR, O'brien DP, Warner BW. A serum factor after intestinal resection stimulates epidermal growth factor receptor signaling and proliferation in intestinal epithelial cells. *Surgery* 2002; **132**: 377-383

109 **Yang J**, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release

of cytochrome c from mitochondria blocked. *Science* 1997; **275**: 1129-1132
110 **Hu Y**, Benedict MA, Wu D, Inohara N, Núñez G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci USA* 1998; **95**: 4386-4391
111 **Stephens J,** Stoll B, Guan XF, Helmrath M, Burrin D. Relative stimulation of superior mesenteric arterial (SMA) blood flow by enteral nutrition and glucagon-like peptide 2 (GLP-2) in piglets. *Gastroenterology* 2004; **126**: A142
112 **Guan X**, Stoll B, Lu X, Tappenden KA, Holst JJ, Hartmann B, Burrin DG. GLP-2-mediated up-regulation of intestinal blood flow and glucose uptake is nitric oxide-dependent in TPN-fed piglets 1. *Gastroenterology* 2003; **125**: 136-147
113 **Adrian TE**, Thompson JS, Quigley EM. Time course of adaptive regulatory peptide changes following massive small bowel resection in the dog. *Dig Dis Sci* 1996; **41**: 1194-1203
114 **Martin Del Olmo JC**, Carbajo Caballero MA, Blanco Alvarez JI, Audivert Mena L, Vaquero Puerta C. Decrease in plasma levels of vasoactive intestinal polypeptide in short bowel syndrome: experimental study. *Int Surg*; **83**: 150-153115
Lezoche E, Carlei F, Vagni V, Mora GV, Speranza V. Elevated plasma levels of vasoactive intestinal polypeptide in short bowel syndrome. *Am J Surg* 1983; **145**: 369-370
116 **Andrews NJ**, Irving MH. Human gut hormone profiles in patients with short bowel syndrome. *Dig Dis Sci* 1992; **37**: 729-732
117 **Tatemoto K**. Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. *Proc Natl Acad Sci USA* 1982; **79**: 2514-2518
118 **Tatemoto K**, Mutt V. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature* 1980; **285**: 417-418
119 **El-Salhy M**, Grimelius L, Wilander E, Ryberg B, Terenius L, Lundberg JM, Tatemoto K. Immunocytochemical identification of polypeptide YY (PYY) cells in the human gastrointestinal tract. *Histochemistry* 1983; **77**: 15-23
120 **El-Salhy M**, Wilander E, Grimelius L, Terenius L, Lundberg JM, Tatemoto K. The distribution of polypeptide YY (PYY) - and pancreatic polypeptide (PP) - immunoreactive cells in the domestic fowl. *Histochemistry* 1982; **75**: 25-30
121 **Greeley GH Jr**, Jeng YJ, Gomez G, Hashimoto T, Hill FL, Kern K, Kurosky T, Chuo HF, Thompson JC. Evidence for regulation of peptide-YY release by the proximal gut. *Endocrinology* 1989; **124**: 1438-1443
122 **Ali-Rachedi A**, Varndell IM, Adrian TE, Gapp DA, Van Noorden S, Bloom SR, Polak JM. Peptide YY (PYY) immunoreactivity is co-stored with glucagon-related immunoreactants in endocrine cells of the gut and pancreas. *Histochemistry* 1984; **80**: 487-491
123 **Böttcher G**, Alumets J, Håkanson R, Sundler F. Co-existence of glicentin and peptide YY in colorectal L-cells in cat and man. An electron microscopic study. *Regul Pept* 1986; **13**: 283-291
124 **Savage AP**, Gornacz GE, Adrian TE, Ghatei MA, Goodlad RA, Wright NA, Bloom SR. Is raised plasma peptide YY after intestinal resection in the rat responsible for the trophic response? *Gut* 1985; **26**: 1353-1358
125 **Suzuki T**, Nakaya M, Itoh Z, Tatemoto K, Mutt V. Inhibition of interdigestive contractile activity in the stomach by peptide YY in Heidenhain pouch dogs. *Gastroenterology* 1983; **85**: 114-121
126 **Allen JM**, Fitzpatrick ML, Yeats JC, Darcy K, Adrian TE, Bloom SR. Effects of peptide YY and neuropeptide Y on gastric emptying in man. *Digestion* 1984; **30**: 255-262
127 **Adrian TE**, Savage AP, Fuessl HS, Wolfe K, Besterman HS, Bloom SR. Release of peptide YY (PYY) after resection of small bowel, colon, or pancreas in man. *Surgery* 1987; **101**: 715-719
128 **Wøjdemann M**, Wettergren A, Hartmann B, Holst JJ. Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. *Scand J Gastroenterol* 1998; **33**: 828-832
129 **Wøjdemann M**, Wettergren A, Hartmann B, Hilsted L, Holst JJ. Inhibition of sham feeding-stimulated human gastric acid secretion by glucagon-like peptide-2. *J Clin Endocrinol Metab* 1999; **84**: 2513-2517
130 **Shibata C**, Naito H, Jin XL, Ueno T, Funayama Y, Fukushima K, Hashimoto A, Matsuno S, Sasaki I. Effect of glucagon, glicentin, glucagon-like peptide-1 and -2 on interdigestive gastroduodenal motility in dogs with a vagally denervated gastric pouch. *Scand J Gastroenterol* 2001; **36**: 1049-1055
131 **Ljungmann K**, Hartmann B, Kissmeyer-Nielsen P, Flyvbjerg A, Holst JJ, Laurberg S. Time-dependent intestinal adaptation and GLP-2 alterations after small bowel resection in rats. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G779-G785
132 **Sigalet DL**, Martin GR. Mechanisms underlying intestinal adaptation after massive intestinal resection in the rat. *J Pediatr Surg* 1998; **33**: 889-892
133 **Hill FL**, Zhang T, Gomez G, Greeley GH Jr. Peptide YY, a new gut hormone (a mini-review). *Steroids* 1991; **56**: 77-82
134 **McCormack SA**, Johnson LR. Role of polyamines in gastrointestinal mucosal growth. *Am J Physiol* 1991; **260**: G795-G806
135 **Thomas T**, Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* 2001; **58**: 244-258
136 **Morgan DM**. Polyamines. An overview. *Mol Biotechnol* 1999; **11**: 229-250
137 **Thomas T**, Kiang DT. Additive growth-inhibitory effects of DL-alpha-difluoromethylornithine and antiestrogens on MCF-7 breast cancer cell line. *Biochem Biophys Res Commun* 1987; **148**: 1338-1345
138 **Johnson LR**, McCormack SA. Healing of Gastrointestinal Mucosa: Involvement of Polyamines. *News Physiol Sci* 1999; **14**: 12-17
139 **Han X**, Kazarinoff MN, Seiler N, Stanley BA. Rat colon ornithine and arginine metabolism: coordinated effects after proliferative stimuli. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G389-G399
140 **Seidel ER**, Scemama JL. Gastrointestinal polyamines and regulation of mucosal growth and function. *J Nutr Biochem* 1997; **8**: 104-111
141 **Wang JY**, Johnson LR, Tsai YH, Castro GA. Mucosal ornithine decarboxylase, polyamines, and hyperplasia in infected intestine. *Am J Physiol* 1991; **260**: G45-G51
142 **Gobert AP**, Cheng Y, Akhtar M, Mersey BD, Blumberg DR, Cross RK, Chaturvedi R, Drachenberg CB, Boucher JL, Hacker A, Casero RA Jr, Wilson KT. Protective role of arginase in a mouse model of colitis. *J Immunol* 2004; **173**: 2109-2117
143 **Meleagros L**, Ghatei MA, Bloom SR. Release of vasodilator, but not vasoconstrictor, neuropeptides and of enteroglucagon by intestinal ischaemia/reperfusion in the rat. *Gut* 1994; **35**: 1701-1706
144 **Pillai RB**, Tolia V, Rabah R, Simpson PM, Vijesurier R, Lin CH. Increased colonic ornithine decarboxylase activity in inflammatory bowel disease in children. *Dig Dis Sci* 1999; **44**: 1565-1570
145 **Antony T**, Thomas T, Shirahata A, Thomas TJ. Selectivity of polyamines on the stability of RNA-DNA hybrids containing phosphodiester and phosphorothioate oligodeoxyribonucleotides. *Biochemistry* 1999; **38**: 10775-10784
146 **Thulesen J**, Hartmann B, Hare KJ, Kissow H, Ørskov, Holst JJ, Poulsen SS. Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice. *Gut* 2004; **53**: 1145-1150
147 **Hamm-Alvarez SF**. Focus on "EGF receptor downregulation depends on a trafficking motif in the distal tyrosine kinase domain". *Am J Physiol Cell Physiol* 2002; **282**: C417-C419
148 **Levkowitz G**, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B, Yarden Y. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* 1998; **12**: 3663-3674

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www.wjgnet.com
wjg@wjgnet.com

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GASTRIC CANCER

Alphastatin downregulates vascular endothelial cells sphingosine kinase activity and suppresses tumor growth in nude mice bearing human gastric cancer xenografts

Lin Chen, Tao Li, Rong Li, Bo Wei, Zheng Peng

Lin Chen, Tao Li, Rong Li, Bo Wei, Zheng Peng, Department of General Surgery, General Hospital of Chinese PLA, Beijing 100853, China
Correspondence to: Professor Lin Chen, Department of General Surgery, General Hospital of Chinese PLA, Beijing 100853, China. chenlinbj@vip.sina.com
Telephone: +86-10-66937846 Fax: +86-10-66937846
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Abstract

AIM: To investigate whether alphastatin could inhibit human gastric cancer growth and furthermore whether sphingosine kinase (SPK) activity is involved in this process.

METHODS: Using migration assay, MTT assay and Matrigel assay, the effect of alphastatin on vascular endothelial cells (ECs) was evaluated *in vitro*. SPK and endothelial differentiation gene (EDG)-1, -3, -5 mRNAs were detected by reverse transcription-polymerase chain reaction (RT-PCR). SPK activity assay was used to evaluate the effect of alphastatin on ECs. Matrigel plug assay in nude mice was used to investigate the effect of alphastatin on angiogenesis *in vivo*. Female nude mice were subcutaneously implanted with human gastric cancer cells (BGC823) for the tumor xenografts studies. Micro vessel density was analyzed in Factor VIII-stained tumor sections by the immunohistochemical SP method.

RESULTS: *In vitro*, alphastatin inhibited the migration and tube formation of ECs, but had no effect on proliferation of ECs. RT-PCR analysis demonstrated that ECs expressed SPK and EDG-1, -3, -5 mRNAs. *In vivo*, alphastatin sufficiently suppressed neovascularization of the tumor in the nude mice. Daily administration of alphastatin produced significant tumor growth suppression. Immunohistochemical studies of tumor tissues revealed decreased micro vessel density in alphastatin-treated animals as compared with controls.

CONCLUSION: Downregulating ECs SPK activity may be one of the mechanisms that alphastatin inhibits gastric cancer angiogenesis. Alphastatin might be a useful and relatively nontoxic adjuvant therapy in the treatment of gastric cancer.

INTRODUCTION

Angiogenesis, the development of new blood vessels from pre-existing endothelium is a critical process in many physiological and pathological conditions including embryonic development, organ regeneration, chronic inflammation and solid tumor growth. The process of formation of new blood vessels is complex and involves several discrete steps, such as ECs spreading, migration, proliferation and morphological differentiation of endothelial cells to form tubes. In the last three decades, considerable research has demonstrated that tumor growth and metastasis requires angiogenesis, and micro vascular endothelial cells recruited by the tumor have become an important second target in cancer therapy[1-3]. This is the major reason why angiogenesis has attracted recent attention in the field of pharmacological research. The key for the development of such an angiostatic therapy is to develop useful angiogenesis inhibitors. A number of research groups have shown that various substances are effective in the inhibition of angiogenesis and/or in the treatment of angiogenic diseases like cancer at the experimental animal model level. Various angiostatic factors such as endostatin, angiostatin and thrombospondin have been identified and can block various steps in the tumor angiogenesis pathway. The application of these antiangiogenic agents for cancer treatment is being evaluated through clinical trials and many new angigenesis inhibitors were reported[4-6]. In previous studies, it was reported that alphastatin (24 amino acids) derived from the amino terminus of human fibrinogen could inhibit the growth of murine colonic adenocarcinoma. Significant reduction in tumor volume was found from the fifth day of administration and maintained for 12 d of intraperitoneal injection[7]. S1P, formed through activation of SPK activity,

is a bioactive sphingolipid metabolite abundantly stored in platelets. Recently, S1P has been targeted for its potential roles in angiogenesis. It may stimulate DNA synthesis and chemotactic motility of ECs, and also induce tube formation of ECs on Matrigel. Further investigation indicated that S1P predominantly induces angiogenesis via endothelial differentiation gene (EDG), a family of Gi protein-coupled receptor. Activation of EDG receptors triggers several signaling pathways by pertussis toxin (PTX)-sensitive Gi protein. Moreover the signaling pathways activated by S1P have been extensively studied in various cell types. All of these proved that S1P may be a novel mechanism during angiogenesis.

In the present study, we attempt to extend the previous study to observe whether alphastatin inhibits human gastric cancer growth. Importantly, we first report that alphastatin suppress angiogenesis through downregulating ECs SPK activity and reducing ECs S1P level.

MATERIALS AND METHODS

Materials

Human umbilical vein ECs (HUVECs) and human gastric cancer cells (BGC823) were obtained commercially from ATCC. High glucose Dulbecco's modified Eagle's medium (DMEM) and bovine serum albumin (BSA, 1 g/L) were obtained from Sigma chemicals (St. Louis Mo, USA). Growth factor reduced (GFR) Matrigel was purchased from Becton Dickinson (San Jose, Calif., USA). Alphastatin was synthesized by New England Biolabs (Beverly, MA) using standard peptide synthesis techniques and purified to more than 95% using high performance liquid chromatography (HPLC). Hepatocyte growth factor (HGF) was purchased from Peprotech EC Ltd. Transwell plate was purchased from Corning Costar (Cambridge, MA).

Endothelial cells migration assay

HUVECs and BGC823 cells were cultured in DMEM containing 100 mL/L FBS and 10 g/L penicillin-streptomycin in a 50 mL/L CO_2 incubator at 37℃. Assessment of ECs migration was performed as recently described with minor modifications[8]. HUVECs were dispersed into homogeneous single cell suspensions after trypsinization. These cells were extensively washed with DMEM containing 1 g/L acid-free BSA and resuspended in the same medium. HUVECs (10^5) were dispersed onto the upper chamber of Transwell compartment with 8 μm pore size filter. The cells were allowed to adhere for 1 h at 37℃. The medium in the lower chamber was removed and replaced by migration medium containing HGF, HGF plus alphastatin or medium alone. Migration was allowed to proceed for 4 h at 37℃. The remaining cells attached to the upper surface of the filters were carefully removed with cotton swabs. Migrated cells were stained with crystal violet and finally examined by light microscopy. The numbers of migrated cells in at least 10 consecutive fields were evaluated and the average was calculated. Data were expressed as the mean ± SD of the number of migrated cells per field in 5 separate experiments.

Endothelial cells proliferation

HUVECs proliferation was assayed as described previously[9] using standard MTT assay. Alphastatin was dissolved in PBS and diluted in DMEM medium. Cells were incubated in their complete medium containing 100 mL/L FBS, the complete medium plus alphastatin or the starvation medium without FBS. The cells were seeded into 96-well micro titer plates at 5×10^7cells/L in the presence of various concentrations of alphastatin for 24, 48, 72, and 96 h. At each time point, a quarter volume of MTT solution (2 g MTT/L phosphate-buffered saline) was added to each well and each plate was incubated for 4 h at 37℃ resulting in an insoluble purple formazan product formation. The medium was aspirated and the precipitates dissolved in 100 μL of dimethyl sulfoxide (DMSO) buffered at PH 10.5. Absorbance at 490 nm was determined using R450 microplate reader (Bio-Rad, USA). Each sample was assayed in five duplicates and repeated at least three times. In order to validate the cytotoxity of alphastatin, the cells were seeded at a density of 5×10^4 cells per well in full growth medium in the absence or presence of alphastatin. After 24 h, the data was collected using CellTiter 96® AQueous One Solution Reagent Kit (Promega, USA).

Endothelial cells tube formation on Matrigel

Endothelial tube formation on Matrigel was conducted as described previously[10]. Matrigel (10 g/L per well) at 4℃ was added to a twenty- four-well plates and then allowed to polymerize at 37℃ for 1 h. HUVECs (5×10^4cells) were seeded on Matrigel in 1 ml of growth medium and various concentrations of alphastatin. The plates were incubated at 37℃ for 18 h. All conditions were triply performed. The formation of tube-like structures by HUVECs was analyzed by fluorescence microscope at × 100 magnification and the total area of tubular structures in five random microscopic fields per well was analyzed using Image tools package 3.0.

Reverse transcription polymerase chain reaction (RT-PCR)

After treated with various dose of alphastatin, total RNA was obtained from HUVECs using the Trizol Reagent kit (Invetrogen, USA). Two μg of total RNA was converted to cDNA by treatment with 100 units of reverse transcriptase and 0.5 μg of oligo-dT primer in 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.1 mol/L DTT and 1 mmol/L dNTP at 42℃ for 1 h. The reaction was terminated by heating at 70℃ for 15 min. Two μL of the cDNA mix was used for enzymatic amplification. Polymerase chain reaction was performed in 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 2.5 units of Ex *Taq* DNA plymerase and 0.1 μmol/L for each of primers such as SPK, EDG-1, EDG-3 and EDG-5. The reaction mixture was repeated for 35 cycles, as heated at 95℃ for 45 s, annealed at 57℃ for 30 s and extended at 72℃ for 60 s. The primers used were 5'ATGCACGAGG TGGTGAACG3' (sense) and 5'GGAGGCAGGTGTCTTGG AAC3' (antisense) for the SPK (426 bp); 5'CCGCAAGAACATTTCCAAG (sense) and 5'ACCCACCAACACCCGACAC (antisense) forEDG-1 (608 bp); 5'CCTGC GGGAGCATTA CCA

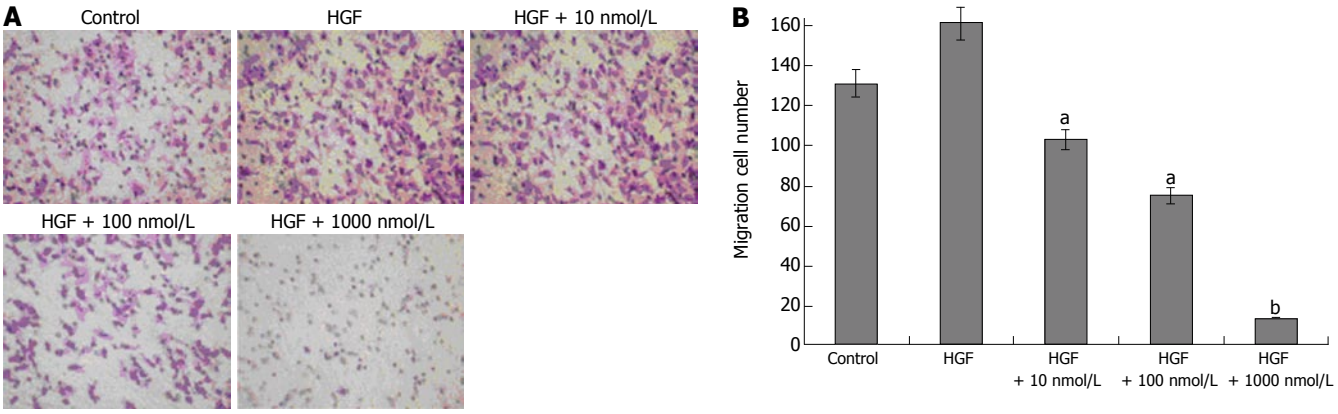

Figure 1 Alphastatin inhibit HUVECs migration induced by HGF(crystatl violet stain, × 100). All data shown as mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* control group.

(sense) and 5'CACCTTACGGCTGCTGGAC (antisense) for EDG-3 (637 bp); 5'AAGTTCCACTCGGCAATGTAC (sense) and 5'GCAGC CAGCAGACGA TAAA (antisense) for EDG-5 (556 bp).

Measurement of sphingosine kinase activity

After various treatments, HUVECs were washed twice with PBS and harvested by scraping in 0.1 mol/L Tris-HCl buffer (pH 7.4) containing 200 mL/L (v/v) glycerol, 1 mmol/L mercaptoethanol, 1 mmol/L EDTA, 1mmol/L Na_3VO_4, 15 mmol/L NaF, 10 mg/L leupeptin and aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride and 0.5 mmol/L 4-deoxypyridoxine. Cells were lysed by freezing and thawing. Cytosolic fractions were prepared by centrifugation at 12000 g for 30 min. One hundred and eighty milliliters of cytosol was incubated with 10 μL of sphingosine (1 mmol/L, dissolved in 50 g/L Triton X-100), 10 μL [γ-^{32}P] ATP (20 mmol/L) containing $MgCl_2$ (200 mmol/L) for 15 min. Sphingosine kinase activity was measured as previously described[11-13].

In vivo Matrigel plug assay

Matrigel plug assay was performed as previously described[14]. Briefly, nude mice were injected subcutaneously with 0.5 mL Matrigel. The injected Matrigel rapidly formed a single solid gel plug. After 7 d, the mice were euthanasia killed and Matrigel plug were removed. It was fixed with 50 g/L formaldehyde phosphate buffer saline and embedded in paraffin. The slides were routinely cut and stained with hematoxylin & eosin (HE). Capillaries were defined as tubular structures containing red blood cells.

Tumor cells implantation and pathology

The in-house and governmental animal protection committees approved all of the experiments and the animals were cared according to the guidelines for laboratory animals established by the Chinese government. Female athymic mice (Balb/c, nu/nu, 8 wk of age; weighing about 20 g) were maintained under clean room conditions in sterile rodent micro isolated cages. Animals received sterile rodent chow and water *ad libitum*. Human gastric cancer cells (BGC 823) were injected subcutaneously (SQ) into the right hind limb (2×10^6 cells in 100 μL of PBS). Tumor cell grew for 8 d until the tumor block established. Animals were randomized for therapy when tumor volume reached 120-160 mm^3. Tumor volume was determined three times weekly by direct measurement with calipers and was calculated by the formula "Volume (V) = length × width^2 × 0.52". Animals were intraperitoneally daily injected with alphastatin (0.25 mg or 2.5 mg/kg per day in 7 mice respectively) or PBS (control in 7 mice). The observations were terminated for ethical reasons when the tumor volume became large compared with the animal size. Tumor for histological analysis was harvested from three animals at d 12 after the start of antiangiogenic therapy. At the end of observation, tumor tissues were fixed in buffered formalin and were embedded in paraffin. Tissue slices (5 μm) were cut and stained with HE. To assess the tumor angiogenesis, immunohistochemical staining was performed using the anti-mouse Factor-VIII monoclonal antibody. Tumor angiogenesis was quantified by counting the number of positively stained microvessels in 10 randomly chosen fields at × 400 magnifications.

Statistical analysis

The data were presented as mean ± SD and analyzed by a statistical software of SPSS 10.0 for Windows program using Student's t-test and the analysis of variance (ANOVA). Significant differences were considered when $P < 0.05$.

RESULTS

Vascular endothelial cell migration and proliferation

Vascular endothelial cell migration is critical for tumor angiogenesis. To determine the effects of alphastatin on migration of HUVECs induced by chemoattractant media (HGF), we counted the number of cells that had migrated to the bottom of the Transwell membrane. Alphastatin significantly inhibited HUVECs migration in response to HGF in a dose-dependent manner (103 ± 4 and 75 ± 3 *vs* 131 ± 4, $P < 0.05$; 13 ± 1 *vs* 131 ± 4, $P < 0.01$, Figure 1). Alphastatin had no effect on cell proliferation even up to 2000 nmol/L for 96 h (Figure 2) .The cytotoxicity of alphastatin was assessed and no detectable cytotoxic effect at those doses was found *in vitro* (data not shown).

Tube formation by HUVECs

When HUVECs were placed on growth factor-reduced

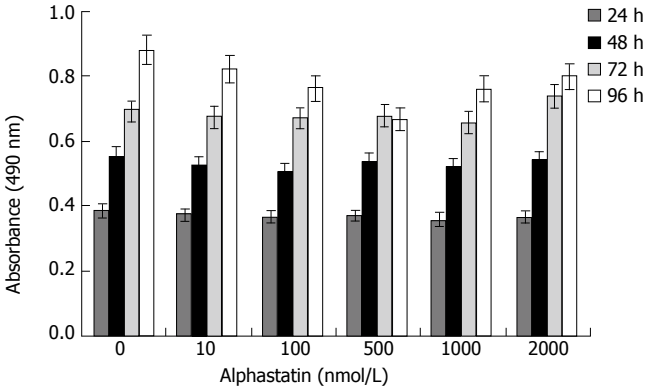

Figure 2 Alphastatin has no effect on HUVECs proliferation.

Matrigel surface, they formed a branching and anastomosing network of capillary like tubules with multicentric junctions over 18 h. Alphastatin inhibited the formation of tubular networks induced by PMA (25 μg/L) on Matrigel in a dose-dependent manner (Figure 3). A remarkable inhibition of tube formation was observed in the presence of 100 nmol/L (1508.96 ± 29.89 μm^2 *vs* 2150 ± 31.05 μm^2, $P < 0.05$). At the same size field, there was longer distance between tubes and less tubules at alphastatin-treated group. Tube formation was quantitatively estimated by measuring the area covered by the tube network using the image analysis program.

SPK mRNA, EDG-1, EDG-3, and EDG-5 expression

Before measurement of SPK activity, RT-PCR was carried out using specific primers for SPK, EDG-1, EDG-3 and EDG-5 to assure expression of them in HUVECs. It was revealed that SPK, EDG-1, EDG-3 and EDG-5 were expressed in HUVECs (Figure 4). Whether alphastatin could change HUVECs SPK activity is a mistery. HUVECs were starved overnight in serum-free medium before the addition of alphastatin. Then, these cells were stimulated with various concentration of alphastatin for different times. Alphastatin could downregulate SPK in a dose-dependent manner. When HUVECs were treated with alphastatin at a concentration of 100 nmol/L, the cellular SPK activity reached a minimum value at 6 h (Figure 5). These data showed that alphastatin leads to downregulation of SPK through stimulation of HUVECs.

New capillaries formation

Matrigel was injected into nude mice with or without alphastatin. Reduction in the number of blood vessels was observed at 100 nmol/L alphastatin group (Figure 6). Sections of experimental tumors were stained with antimouse Factor-VIII antibody.

Microvessel density was less in alphastatin-treated group than in PBS group (Figure 7).

Gastric cancer growth in nude mice

For tumor growth quantification, we used the human BGC823 gastric cancer cell lines. After injecting tumor cells, tumors were allowed to grow until the block established (10 d).Then mice were randomized and divided into control group (PBS: $n = 7$) and therapy group

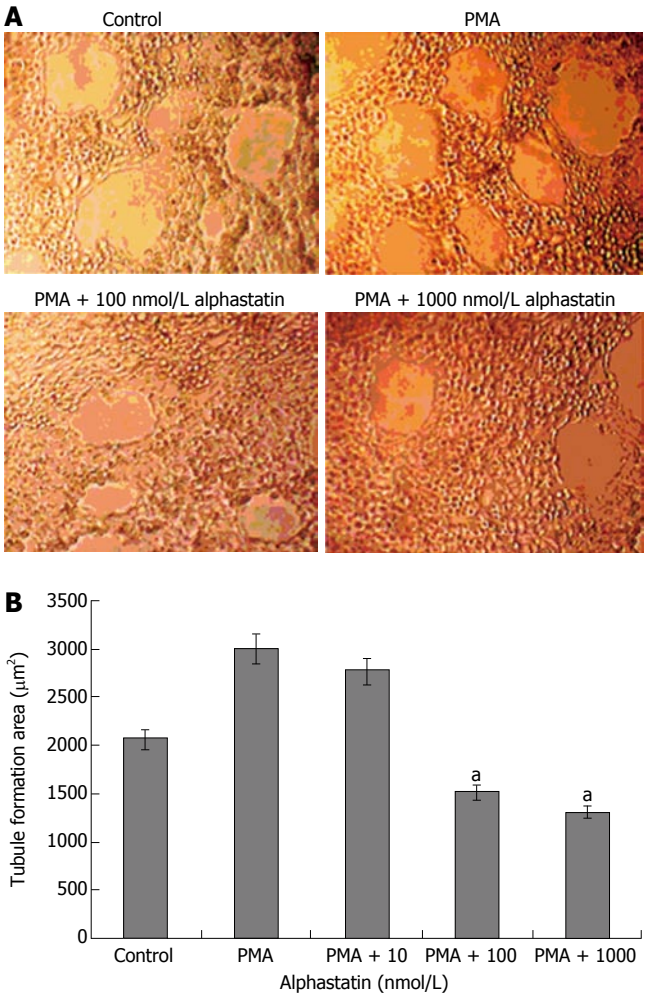

Figure 3 Alphastatin inhibit HUVECs tubule formation in response to PMA. All data shown as mean ± SD. $^aP < 0.05$ *vs* control group.

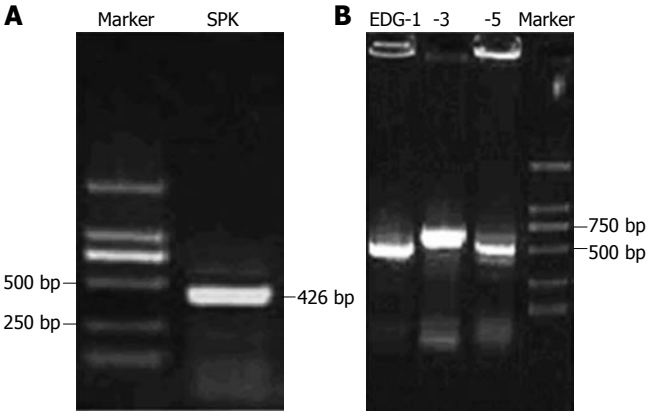

Figure 4 Expression of SPK , EDG-1, EDG-3, EDG-5 in HUVECs.

(alphastatin: $n = 7$). The tumor in control group steadily grew up to a final tumor volume of 1771 ± 262 mm^3 over the same period as the therapy group. However, the growth rate of the tumor in the therapy group was significantly reduced between d 18 and d 26 ($P < 0.05$ and $P < 0.01$, Figure 8), and a final tumor volume was only 1145 ± 114 mm^3 and 612 ± 173 mm^3 respectively. Moreover, alphastatin injection appeared to be well tolerated *in vivo*

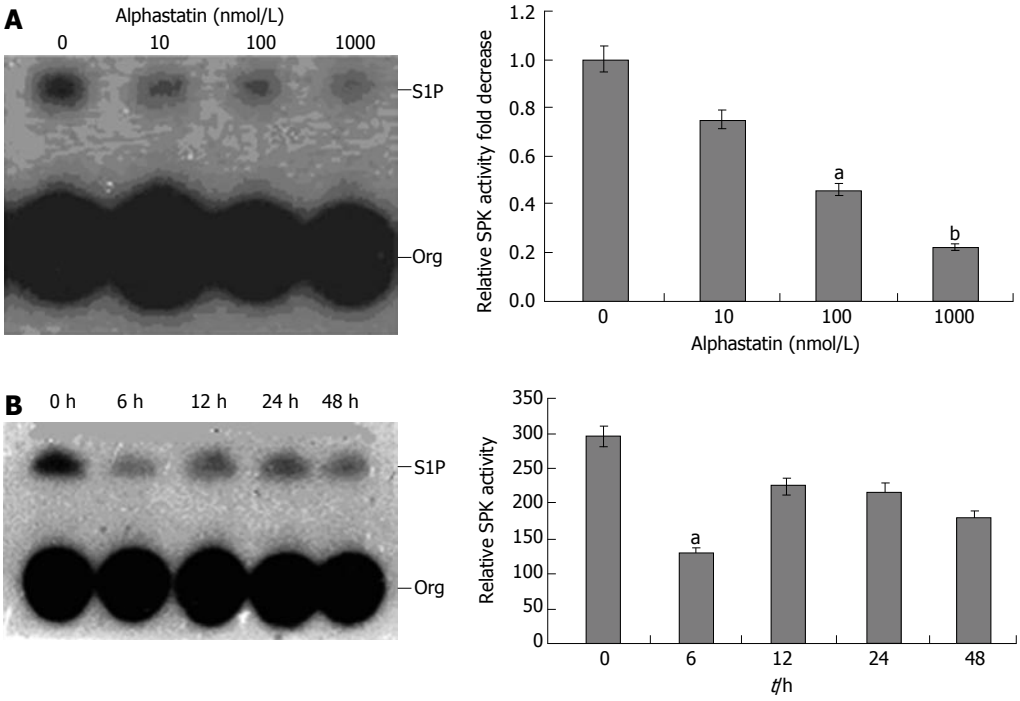

Figure 5 Alphastatin downregulate HUVECs SPK activity in a dose-dependent manner. HUVECs SPK activity reached a minimum value at 6 h. All data shown as mean ± SD. **A**: $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* 0 nmol/L alphastatin; **B**: $^{a}P < 0.05$ *vs* 0 h group.

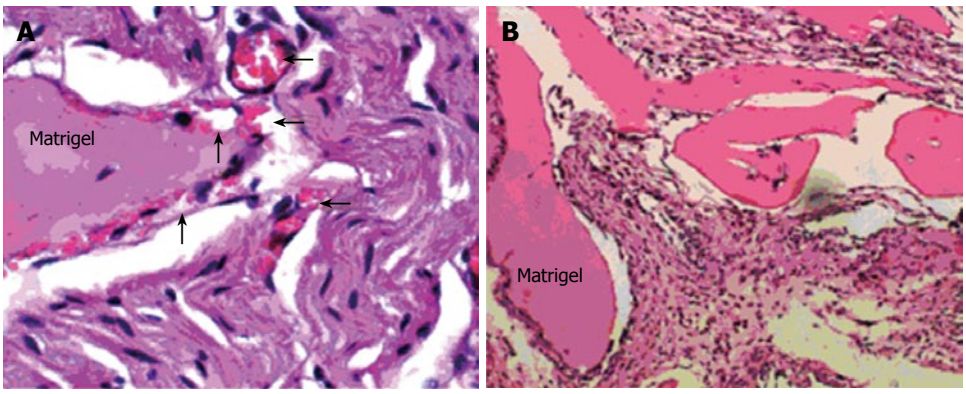

Figure 6 Alphastatin inhibit new capillaries formation on Matrigel plug (original magnification × 100). **A**: Many newly formed capillaries full of red blood cells (arrow); **B**: Granuloma formation without significant neovasculature.

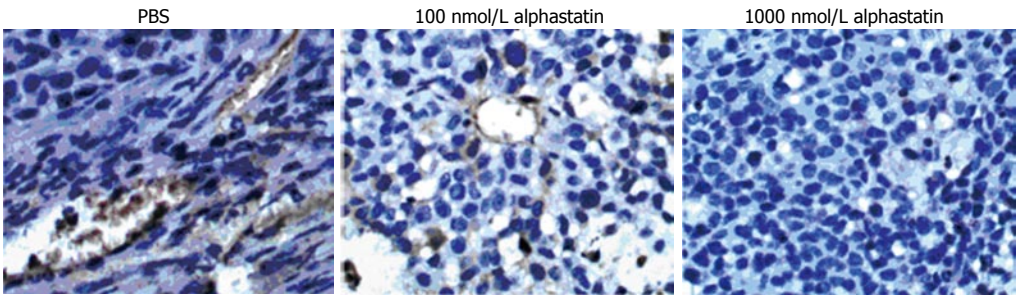

Figure 7 Factor-VIII staining revealed decreased blood vessel density in alphastatin-treated group *vs* PBS group (× 400).

and had no significant effect on animal body mass and general condition.

DISCUSSION

Previous studies show that alphastatin is a potent antiangiogenic agent[7]. However, little is known about whether it has any effect on human derived tumors and what mechanism related to the antiangiogenic activity of alphastatin. In the present study, we therefore tried to solve the question above. Gastric cancer is one of the most common malignancies in Asia. However, so far there is no effective therapeutic measure for this highly malignant disease. Recent studies have demonstrated that angiogenesis is a prerequisite for development and growth of different tumors[15-18] Anti-angiogenic targeting of the neovasculature within tumors is considered one of the most promising strategies in the search for novel antineoplastic therapies[19]. Strategies for anti-angiogenic drugs were described as follows: (1) interference with endothelial cell migration and proliferation; (2) interference with endothelial cell tubule formation and (3)

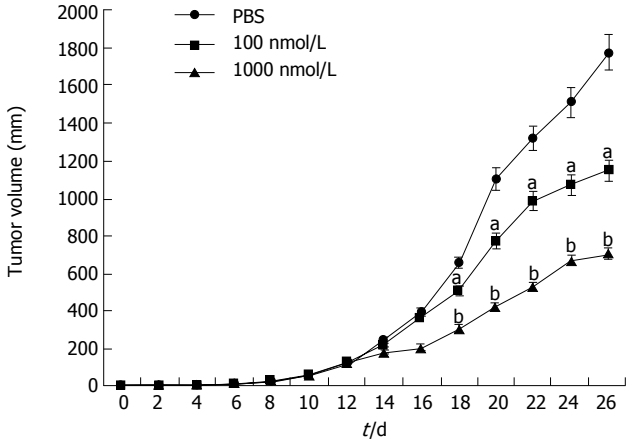

Figure 8 Animals injected with alphastatin exhibited a significantly reduced tumor volume. All data shown as mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* PBS group.

interference with some factors involved in angiogenesis pathway[20-25]. The present study shows that alphastatin inhibits angiogenesis *in vitro* and *in vivo*. Furthermore, for the first time we described that alphastatin could suppress human gastric cancer growth in nude mice through downregulating vascular endothelial cell SPK activity. In migration assay, we chose HGF as chemoattractant media because it is a multifunctional factor regulating cell growth, motility and migration for a variety of cell types including endothelial cell, epithelial cell and hepatocyte. Its effect of chemotaxis is greater than vascular endothelial growth factor (VEGF). Duan *et al* showed that HGF/c-Met activates SPK *via* ERK1/2 and PI3K pathways. SPK activation plays an important regulatory role in HGF-induced migration of endothelial cells[26]. Lee *et al* showed that S1P strongly induces the formation of tube network of HUVECs which has the characteristics of strong intercellular interaction and sustained survival of large population of cells on Matrigel[11]. These morphological changes of HUVECs may occur mainly through binding of S1P with EDG-1. In addition, S1P in part and indirectly stimulates HUVECs proliferation through direct secretion of angiogenic factors, because an increase in the expression of VEGF mRNA was detected within 24 h after treatment of HUVECs with S1P[27-29]. In the present study, alphastatin inhibited the migration and tube formation of HUVECs, but no significant effect on cell proliferation even up to 2000 nmol/L. It was in SPK activity assay shown that alphastatin down regulated HUVECs SPK activity and reduced cellular S1P production. From the data above, it could be concluded that alphastatin inhibited the process of angiogenesis through interference with HUVECs SPK activity.

In the *in vivo* Matrigel plug assay, there was only granuloma formation without significant neovasculature in alphastatin group. Therefore, the antiangiogenic action of alphastatin *in vivo* may be due to its inhibitory effects on endothelial cells stimulated by VEGF as shown in the present study[5]. In our tumor model, the BGC823 tumors continued to grow over the 12-d injection period, whereas nude mice injected daily with alphastatin demonstrated significant reduction in tumor volume from d 8 of administration and this was maintained for 14 d of injection. The inhibition was in a dose-dependent manner. Immunohistochemical studies of tumor tissues revealed decreased microvessel density in alphastatin-treated animals as compared with control group. It is also likely that alphastatin down regulated SPK activity and reduced endothelial cell S1P production, resulted in inhibition of angiogenesis.

In conclusion, our current studies provide evidence for anti-angiogenic activities of alphastatin. It induced human gastric cancer vascular growth ceasing *in vivo*, inhibited tube formation on Matrigel and disrupted HUVECs migration. More importantly, the inhibition of angiogenesis was through down regulating HUVECs SPK activity and this may be a novel mechanism that alphastatin exerts antiangiogenic activity. All of these findings hint that, as an new anti-angiogenic agent, alphastatin might be a prototype anti-tumor drug.

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REFERENCES

1 **Carmeliet P**, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249-257
2 **Bamias A**, Dimopoulos MA. Angiogenesis in human cancer: implications in cancer therapy. *Eur J Intern Med* 2003; **14**: 459-469
3 **Ruoslahti E**. Specialization of tumour vasculature. *Nat Rev Cancer* 2002; **2**: 83-90
4 **Aoki K**, Watanabe K, Sato M, Ikekita M, Hakamatsuka T, Oikawa T. Effects of rhizoxin, a microbial angiogenesis inhibitor, on angiogenic endothelial cell functions. *Eur J Pharmacol* 2003; **459**: 131-138
5 **Jansen M**, de Witt Hamer PC, Witmer AN, Troost D, van Noorden CJ. Current perspectives on antiangiogenesis strategies in the treatment of malignant gliomas. *Brain Res Brain Res Rev* 2004; **45**: 143-163
6 **Bikfalvi A**, Bicknell R. Recent advances in angiogenesis, anti-angiogenesis and vascular targeting. *Trends Pharmacol Sci* 2002; **23**: 576-582
7 **Staton CA**, Brown NJ, Rodgers GR, Corke KP, Tazzyman S, Underwood JC, Lewis CE. Alphastatin, a 24-amino acid fragment of human fibrinogen, is a potent new inhibitor of activated endothelial cells in vitro and in vivo. *Blood* 2004; **103**: 601-606
8 **Tarui T**, Majumdar M, Miles LA, Ruf W, Takada Y. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. *J Biol Chem* 2002; **277**: 33564-33570
9 **Sahni A**, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. *Blood* 2000; **96**: 3772-3778
10 **Guidolin D**, Vacca A, Nussdorfer GG, Ribatti D. A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay in vitro. *Microvasc Res* 2004; **67**: 117-124
11 **Lee OH**, Kim YM, Lee YM, Moon EJ, Lee DJ, Kim JH, Kim KW, Kwon YG. Sphingosine 1-phosphate induces angiogenesis: its angiogenic action and signaling mechanism in human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 1999; **264**: 743-750
12 **Melendez AJ**, Carlos-Dias E, Gosink M, Allen JM, Takacs L. Human sphingosine kinase: molecular cloning, functional

characterization and tissue distribution. *Gene* 2000; **251**: 19-26
13 **McVerry BJ**, Garcia JG. In vitro and in vivo modulation of vascular barrier integrity by sphingosine 1-phosphate: mechanistic insights. *Cell Signal* 2005; **17**: 131-139
14 **Phongkitkarun S**, Kobayashi S, Kan Z, Lee TY, Charnsangavej C. Quantification of angiogenesis by functional computed tomography in a Matrigel model in rats. *Acad Radiol* 2004; **11**: 573-582
15 **Siemann DW**, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. *Cancer* 2004; **100**: 2491-2499
16 **Nicolella D**, Maione P, Gridelli C. Targeted therapies: focus on a new strategy for gastrointestinal tumors. *Crit Rev Oncol Hematol* 2003; **47**: 261-271
17 **Tozer GM**. Measuring tumour vascular response to antivascular and antiangiogenic drugs. *Br J Radiol* 2003; **76** Spec No 1: S23-S35
18 **Baluk P**, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* 2005; **15**: 102-111
19 **Griffioen AW**, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* 2000; **52**: 237-268
20 **Alessi P**, Ebbinghaus C, Neri D. Molecular targeting of angiogenesis. *Biochim Biophys Acta* 2004; **1654**: 39-49
21 **Gupta MK**, Qin RY. Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* 2003; **9**: 1144-1155
22 **Zhang HT**, Bicknell R. Therapeutic inhibition of angiogenesis. *Mol Biotechnol* 2003; **25**: 185-200
23 **Brack SS**, Dinkelborg LM, Neri D. Molecular targeting of angiogenesis for imaging and therapy. *Eur J Nucl Med Mol Imaging* 2004; **31**: 1327-1341
24 **Liekens S**, De Clercq E, Neyts J. Angiogenesis: regulators and clinical applications. *Biochem Pharmacol* 2001; **61**: 253-270
25 **Bicknell R**. The realisation of targeted antitumour therapy. *Br J Cancer* 2005; **92** Suppl 1: S2-S5
26 **Duan HF**, Wu CT, Lu Y, Wang H, Liu HJ, Zhang QW, Jia XX, Lu ZZ, Wang LS. Sphingosine kinase activation regulates hepatocyte growth factor induced migration of endothelial cells. *Exp Cell Res* 2004; **298**: 593-601
27 **Allende ML**, Proia RL. Sphingosine-1-phosphate receptors and the development of the vascular system. *Biochim Biophys Acta* 2002; **1582**: 222-227
28 **Shu X**, Wu W, Mosteller RD, Broek D. Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. *Mol Cell Biol* 2002; **22**: 7758-7768
29 **Ren J**, Dong L, Xu CB, Pan BR. Expression of sphingosine kinase gene in the interactions between human gastric carcinoma cell and vascular endothelial cell. *World J Gastroenterol* 2002; **8**: 602-607

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
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Effects of inositol hexaphosphate on proliferation of HT-29 human colon carcinoma cell line

Ying Tian, Yang Song

Ying Tian, Yang Song, Qingdao University Medical College, 38 Dengzhou Road, Qingdao 266021, Shandong Province, China
Supported by Qingdao Science and Technology Bureau (to Yang Song)
Correspondence to: Professor Yang Song, Qingdao University Medical College, 38 Dengzhou Road, Qingdao 266021, Shandong Province, China. qdsongyang@126.com
Telephone: +86-532-82991029
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Abstract

AIM: To investigate the effects of inositol hexaphosphate (IP_6) on proliferation of HT-29 human colon carcinoma cell line.

METHODS: Cells were exposed to various concentrations (0, 1.8, 3.3, 5.0, 8.0, 13.0 mmol/L) of IP_6 for a certain period of time. Its effect on growth of HT-29 cells was measured by MTT assay. The expressions of cell cycle regulators treated with IP_6 for 2 d were detected by immunocytochemistry.

RESULTS: IP_6 inhibited the HT-29 cell growth in a dose- and time-dependent manner. Analysis of cell cycle regulator expression revealed that IP_6 reduced the abnormal expression of P53 and PCNA and induced the expression of P21.

CONCLUSION: IP_6 has potent inhibitory effect on proliferation of HT-29 cells by modulating the expression of special cell cycle regulators.

Key words: Phytic Acid; Colonic neoplasms; Cell proliferation

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INTRODUCTION

Colorectal cancer is the second most frequent cancer in Western countries[1], and the third leading cause of cancer deaths in the United States[2]. In China, the mortality rate of colorectal cancer is the fourth to sixth leading cause of cancer deaths[3]. Epidemiological studies have shown that high fiber foods, such as fruits, vegetables, whole grains and cereals, may protect against colorectal cancer[4-8]. Animal studies have shown that wheat bran has protective effect against colorectal cancer[9-14], which is attributed mostly to its high fiber content. Interestingly, many of the proposed protective mechanisms of wheat bran fiber, such as decreased transit time[15], increased bulk[16] and fermentation[17], are analogous to those of inositol hexaphosphate (IP_6 or phytic acid), which is a major fiber-associated component of wheat bran[18]. In some epidemiological studies, colorectal cancer-protective effect of fiber foods rich in IP_6, such as wheat bran has been observed[18], indicating that IP_6 may protect against colorectal cancer.

IP_6 is a naturally occurring polyphosphorylated carbohydrate, found in plants, particularly in cereals and legumes (0.4%-6.4%)[19]. It consists of a myo-inositol ring with six dihydrogen phosphate groups, assuming a chair conformation in dilute solution[20]. This unique structure empowers IP_6 with a chelating capacity of binding to polyvalent (both mono and divalent) cations. Some of these metal ions such as magnesium and zinc play an important role in stimulation of cellular proliferation[21]. This molecule is related to human health as an anti-nutrient. However, during the last decades it has been shown that IP_6 is also widely distributed in animal cells and tissues at substantial levels[22,23]. Especially, a strong anti-cancer activity of IP_6 has been demonstrated both *in vivo* and *in vitro*[24]. IP_6 exerts its anti-cancer activity by entering into cellular inositol hexaphosphate pool and affecting common cellular signal transduction pathways[24,25], but its mechanisms of action are still not completely understood.

This study was to examine the effect of IP_6 on growth of HT-29 human colon carcinoma cell line. The expressions of cell cycle regulators were assessed after IP_6 treatment.

MATERIALS AND METHODS

Chemicals

IP_6 (a dodecasodium salt from rice) and 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) were purchased from Sigma (St Louis, MO, USA). DMEM/Ham F12 culture medium, fetal bovine serum and trypsin were from Gibco BRL (Grand Island, NY, USA). Rabbit polyclonal antibody to human P53, mouse monoclonal antibodies to human P21, PCNA and SP

histostain-plus kits were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines, culture conditions and IP_6 treatment

The HT-29 human colon carcinoma cell line was obtained from Xiehe Medical University (Beijing, China). Cells were grown in DMEM/Ham F12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 g/L) under standard culture conditions (37℃, 950 mL/L humidified air and 50 mL/L CO_2). A stock solution of 100 mmol/L IP_6 in distilled water was freshly prepared each time before use, the pH was neutralized with NaOH and sterilized by passing through a 0.22 μmol/L membrane filter. Dilutions of 1.8, 3.3, 5.0, 8.0, 13.0 mmol/L in DMEM/Ham F12 medium were prepared from stock solution immediately before use. The DMEM/Ham F12 medium with equal volume of distilled water served as negative control.

MTT assay

Cell number was determined by colorimetric MTT assay[24]. MTT was dissolved in PBS at 5 g/L, filter-sterilized, diluted in the DMEM/Ham F12 medium, giving a final concentration of 1.0 g/L. For growth assay, cells were plated in 96-well microtiter plates (Costar, Cambridge, MA, USA) at a density of 2×10^3 cells/well of 100 μL media. One of the IP_6 treatments was that cells were exposed to 1.8-13.0 mmol/L IP_6 for 6 h, 12 h, or 24 h, after which IP_6 was removed and cells were grown in media without IP_6. This treatment was given every other day and continued for 6 d. The other IP_6 treatment was that cells were treated with various concentrations of IP_6 continuously for 6 d, during which culture media were changed with fresh media every other day. At the indicated time the media were removed, 50 μL of MTT was added, and the incubation was continued for 4 h at 37℃. Individual cell viability was assessed by visualization of intracellular blue crystal formation by light microscopy. The precipitated formazan was dissolved with 150 μL of DMSO, and the absorbance was determined at 490 nm with a microplate autoreader (EL311sx, Bio-Tek Instruments, Inc., Winooski, VT). Cell growth assay was repeated three times.

Immunocytochemistry

Immunocytochemical staining for P53, P21 and PCNA was carried out by the standard streptavidin-peroxidase-biotin technique (SP technique) using SP kit. Cells were treated with 1.8-13.0 mmol/L IP_6 for 2 d and collected by a brief trypsinization and plated on slides. The cells were fixed in acetone at -20℃ for 5 min. The endogenous peroxidase activity was quenched in a 3% solution of hydrogen peroxide for 15 min and blocked for 10 min. Cells were immunostained with monoclonal antibody (dilutions: P21 1:50, PCNA 1:80) and P53 polyclonal antibody (dilutions: 1:50) for 1 h at 37℃. After three further washes with PBS, a second biotinylated goat anti-rabbit or rabbit anti-mouse antibodiy was applied for 1 h at room temperature and then streptavidin conjugated to peroxidase was added. Following extensive washes with PBS, 3, 3-diaminobenzidine was used for color development, and hematoxylin was used for counterstaining. The negative controls were performed by substituting the primary antibody with PBS. Hematoxylin-stained cells were examined under light microscope and photographed. Cells not counterstained were measured by VIDAS2.1 image analysis system for absorbance because hematoxylin staining could affect the image-analysis results. Three highly magnified visual fields which were not overlapped were randomly selected to measure the absorbance of each field. The mean absorbance was calculated.

Table 1 P53 expression in IP_6-treated HT-29 cells (mean ± SD)

Concentration of IP_6 (mmol/L)	Absorbance value
Control	0.6772 ± 0.0095
1.8	0.6161 ± 0.0203
3.3	0.5996 ± 0.0205
5.0	0.6067 ± 0.0130
8.0	0.5871 ± 0.0159
13.0	0.5817 ± 0.0158

Statistical analysis

The experimental results were repeated three times and expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 11.5 (SPSS Inc, Chicago, IL, USA).

RESULTS

Effect of IP_6 on the growth of HT-29 cells

Continuous treatment with IP_6 inhibited the proliferation of HT-29 cells (Figure 1A). The absorbance value for each IP_6 group was lower than that of control group. At the same time point, the absorbance value decreased with increasing IP_6 concentration. The absorbance values for the 8.0 mmol/L and 13.0 mmol/L IP_6 groups decreased on d 6 ($P < 0.05$). The effects of discontinuous treatment with IP_6 on the growth of HT-29 cells are shown in Figures 1B-1D. The treatment with IP_6 for 24 h inhibited the cell growth. But neither 6 h nor 12 h treatment showed dose- or time-dependent inhibition effects though the absorbance value for each IP_6 group was lower than that for control group.

Effects of IP_6 on the expression of P53, P21 and PCNA

Compared to the control, the expression of P53 protein in HT-29 cells was decreased after 2 d of IP_6 treatment at different concentrations ($P < 0.05$) (Table 1 and Figure 2A, Figure 2B).

Compared to the control, treatment of HT-29 cells with IP_6 at various concentrations for 2 d increased the expression of P21 ($P < 0.05$) (Table 2 and Figure 2C, Figure 2D).

Compared to the control, the expression of PCNA decreased after treated with IP_6 at different concentrations for 2 d ($P < 0.05$) (Table 3 and Figure 2E, Figure 2F).

The absorbance values assayed by MTT after IP_6 treatment for different periods of time are listed in Tables 4, 5 , 6, and 7.

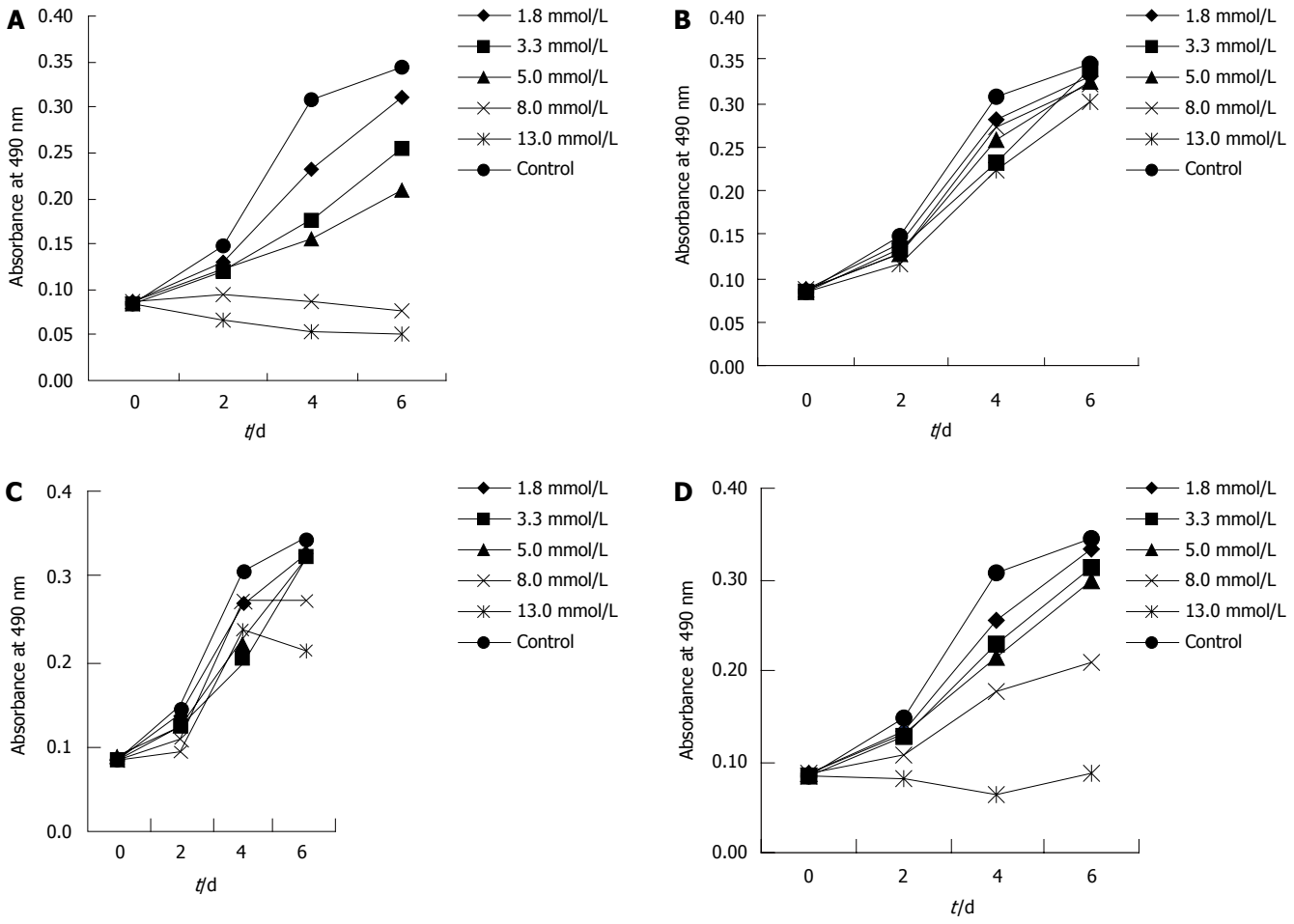

Figure 1 MTT assay showing the effect of IP6 on the growth of HT-29 cells after treated for 0 (**A**), 6 (**B**), 12 (**C**), 24 h (**D**).

Table 2 P21 expression in IP6-treated HT-29 cells (mean ± SD)

Concentration of IP6 (mmol/L)	Absorbance value
Control	0.4868 ± 0.0486
1.8	0.6011 ± 0.0152
3.3	0.5138 ± 0.0336
5.0	0.6032 ± 0.0105
8.0	0.6078 ± 0.0066
13.0	0.5981 ± 0.0163

Table 3 PCNA expression in IP6-treated HT-29 cells (mean ± SD)

Concentration of IP6 (mmol/L)	Absorbance value
Control	0.6407 ± 0.0096
1.8	0.6361 ± 0.0087
3.3	0.5904 ± 0.0302
5.0	0.4520 ± 0.0495
8.0	0.4788 ± 0.0357
13.0	0.5006 ± 0.0403

DISCUSSION

Uncontrolled proliferation is one of the most important characteristics of malignant cells due to the aberrations of cell cycle regulators such as mutation, activation or inactivation of genes. Identification of cell cycle regulator specificity of anti-tumor drugs is essential to understand the mechanisms of their action.

MTT assay in this study showed that the growth of HT-29 cells was inhibited after continuous IP6 treatment for 2-6 d ($P < 0.05$). The effect enhanced with increasing IP6 concentration and prolonged treatment time, suggesting that the inhibition effects of IP6 are dose- and time-dependent.

To confirm our data we used another proliferating marker, proliferating cell nuclear antigen (PCNA) which is essential for both DNA replication and repair[26]. During DNA replication, PCNA forms a ring structure clamping the synthesized DNA to the DNA polymerases δ and ε to ensure continuation of the replication process[27,28]. In case of DNA damage, PCNA binds to the over-expressed $P21^{waf1/cip1}$ leading to inhibition of PCNA-dependent replication, but it does not affect the DNA repair function attained by PCNA[29]. Thus, PCNA is expressed in both cycling and non-cycling cells[30]. Immunocytochemistry in this study showed that IP6-treated cells reduced PCNA expression compared with control cells ($P < 0.05$), although the dose-dependent inhibition was not obvious, which was in agreement to the low proliferation rate observed in MTT assay, indicating that IP6 inhibits proliferation of HT-29 cells.

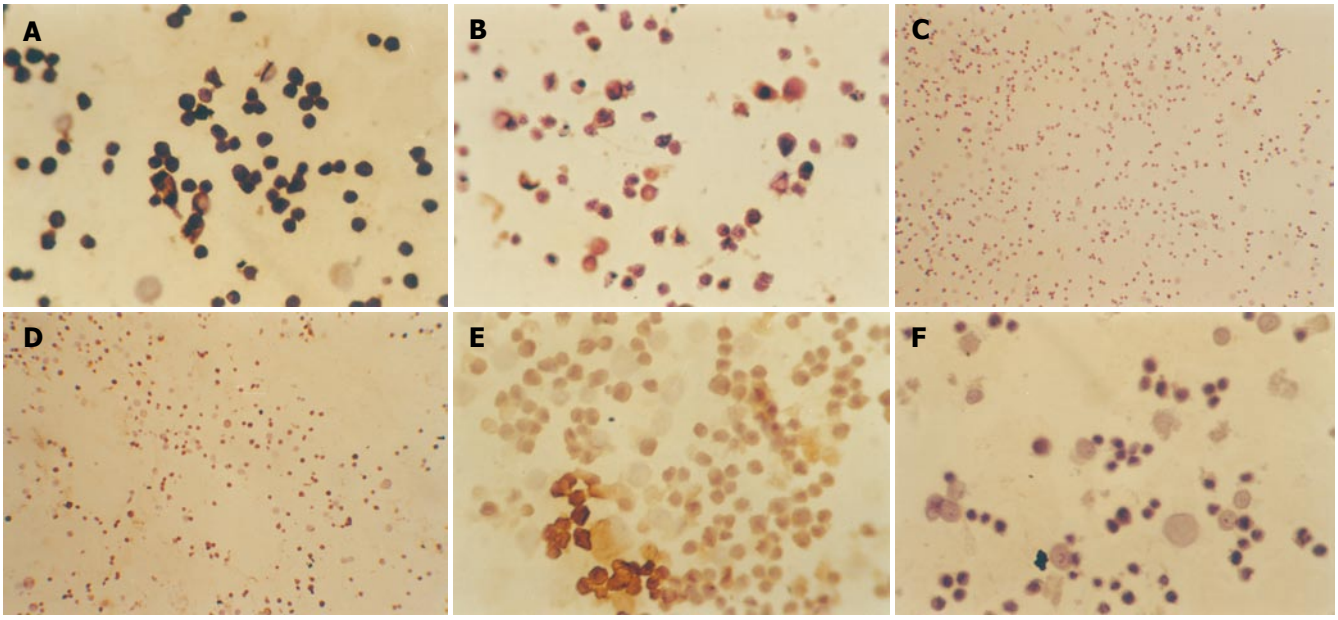

Figure 2 Expression of p53 (**A**, **B**), p21 (**C**, **D**) and PCNA (**E**, **F**) in untreated control and IP6-treated cells. The slides were counterstained with hematoxylin (× 400, × 100, × 400, respectively).

Table 4 Absorbance values assayed by MTT after continuous treatment with IP6 (mean ± SD)

IP6 (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 ± 0.0021	0.1487 ± 0.0031	0.3083 ± 0.0240	0.3450 ± 0.0056
1.8	0.0857 ± 0.0045	0.1293 ± 0.0049	0.2320 ± 0.0155	0.3097 ± 0.0180
3.3	0.0837 ± 0.0042	0.1203 ± 0.0075	0.1770 ± 0.0227	0.2537 ± 0.0300
5.0	0.0873 ± 0.0057	0.1233 ± 0.0029	0.1547 ± 0.0042	0.2077 ± 0.0153
8.0	0.0857 ± 0.0021	0.0940 ± 0.0053	0.0873 ± 0.0032	0.0757 ± 0.0025
13.0	0.0847 ± 0.0032	0.0660 ± 0.0010	0.0530 ± 0.0026	0.0510 ± 0.0036

Table 5 Absorbance values assayed by MTT after IP6 treatment for 6 h (mean ± SD)

IP6 (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 ± 0.0021	0.1487 ± 0.0031	0.3083 ± 0.0240	0.3450 ± 0.0056
1.8	0.0857 ± 0.0045	0.1397 ± 0.0023	0.2823 ± 0.0344	0.3303 ± 0.0080
3.3	0.0837 ± 0.0042	0.1333 ± 0.0015	0.2317 ± 0.0192	0.3383 ± 0.0025
5.0	0.0873 ± 0.0057	0.1277 ± 0.0025	0.2593 ± 0.0131	0.3240 ± 0.0082
8.0	0.0857 ± 0.0021	0.0940 ± 0.0053	0.0873 ± 0.0032	0.0757 ± 0.0025
13.0	0.0847 ± 0.0032	0.1157 ± 0.0035	0.2237 ± 0.0215	0.3020 ± 0.0080

Table 6 Absorbance values assayed by MTT after IP6 treatment for 12 h (mean ± SD)

IP6 (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 ± 0.0021	0.1487 ± 0.0031	0.3083 ± 0.0240	0.3450 ± 0.0056
1.8	0.0857 ± 0.0045	0.1383 ± 0.0065	0.2660 ± 0.0056	0.3267 ± 0.0050
3.3	0.0837 ± 0.0042	0.1230 ± 0.0043	0.1990 ± 0.0554	0.3220 ± 0.0105
5.0	0.0873 ± 0.0057	0.1250 ± 0.0017	0.2290 ± 0.0350	0.3217 ± 0.0076
8.0	0.0857 ± 0.0021	0.1103 ± 0.0038	0.2693 ± 0.0711	0.2723 ± 0.0025
13.0	0.0847 ± 0.0032	0.0957 ± 0.0035	0.2363 ± 0.0299	0.2107 ± 0.0135

Table 7 Absorbance values assayed by MTT after IP6 treatment for 24 h (mean ± SD)

IP6 (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 ± 0.0021	0.1487 ± 0.0031	0.3083 ± 0.0240	0.3450 ± 0.0056
1.8	0.0857 ± 0.0045	0.1323 ± 0.0040	0.2547 ± 0.0153	0.3330 ± 0.0122
3.3	0.0837 ± 0.0042	0.1277 ± 0.0099	0.2300 ± 0.0161	0.3123 ± 0.0115
5.0	0.0873 ± 0.0057	0.1317 ± 0.0032	0.2137 ± 0.0168	0.2990 ± 0.0190
8.0	0.0857 ± 0.0021	0.1067 ± 0.0021	0.1757 ± 0.0116	0.2093 ± 0.0105
13.0	0.0847 ± 0.0032	0.0807 ± 0.0021	0.0650 ± 0.0036	0.0857 ± 0.0051

Since IP6 inhibits cell growth, we studied the regulators of cell cycle. P53, a tumor suppressor protein, is a nuclear transcription factor that controls cell cycle progression[31,32], and plays a role in G1/S check point of cell cycle allowing the repair of damaged DNA[33,34]. Mutations and deletions of the tumor suppressor gene p53 have been identified in about 50% of colorectal carcinomas and are associated with poor prognosis due to its weaker ability to inhibit cell proliferation. The half-life of wild-type P53 is very short and difficult to detect, while the mutant P53 protein has a much longer half life and can be examined by conventional immunohistochemical technology[35]. Rodrigues NR *et al*[36] showed that over-expression of p53 is synonymous with mutation and HT-29 cells have mutations in codon 273 of the p53 gene, so HT-29 cells overproduce mutant p53 antigen. In our study, the immunocytochemical results showed that in IP6-treated cells the abnormal expression of P53 protein decreased compared to control ($P < 0.05$), indicating that IP6 reduces the expression of mutant P53 protein. It was reported that treatment of HT-29 cells with IP6 increases the level of wild-type P53[37]. In the present study, P53 polyclonal antibody was not specified for wild P53 but responded to many antigenic determinants, including mutant P53, indicating that IP6 up-regulates

the expression of wild-type P53 and down-regulates the expression of mutant P53 to control cell cycle check-point and prevent progression of cells to the DNA synthesis phase (S phase) of the cell cycle. But the exact mechanism by which IP_6 affects p53 is not clear and needs further study.

$P21^{waf1/cip1}$ is an inhibitor of cyclin dependent kinases (CDKS) that are required for the cells to enter the S-phase of the cell cycle[38]. The gene encoding $P21^{waf1/cip1}$ is transcriptionally regulated by the protein product of the gene p53. Over-expression of $P21^{waf1/cip1}$ is growth inhibitory, possibly by inhibiting the activity of cyclin/CDK complex[39] which binds to the C-terminal domain of PCNA. The resulting P21-PCNA complex blocks the ability of PCNA to process DNA polymerase in DNA replication. Thus $P21^{waf1/cip1}$ may act as a tumor suppressor because of its role in growth control[39,40]. In the present study, the expression of P21 was increased after IP_6 treatment for 2 d ($P < 0.05$). After counterstaining with hematoxylin, untreated cells were stained purple while IP_6-treated cells were stained yellow, indicating that expression of P21 is higher in IP_6-treated cells. High-expression of $P21^{waf1/cip1}$ leads to decreased nuclear expression of PCNA, which is in agreement with our results.

In summary, IP_6 remarkably inhibits proliferation of HT-29 human colon carcinoma cell line. IP_6 exerts its inhibitory effect in part by affecting special cell cycle regulators and reduces over-expression of mutant P53 and stimulates expression of wild-type P53 and $P21^{waf1/cip1}$. $P21^{waf1/cip1}$ binds to PCNA, thus preventing PCNA-dependent cellular proliferation. In our immunocytochemical experiments, cells grew very slowly and were not adhered in media with high IP_6 dose, fell off and died very soon. The effect of 13.0 mmol/L IP_6 on expression of genes was less than that of 8.0 mmol/L IP_6, partly due to the rapid death of cells in 13.0 mmol/L IP_6, indicating that that IP_6 has no significant effect on the expression of genes. Furthermore, neither significant dose-dependent effect of IP_6 was observed on the expressions of cell cycle regulators nor obvious correlation among these indexes was found, possibly owing to the short period of IP_6 treatment (only 2 d), suggesting that the effects of IP_6 on gene expressions are relatively weak.

The present study is merely a preliminary investigation of IP_6 on colon cancer. The results are also limited although the effects of IP_6 can be seen. Additional research is needed to explore the mechanisms of IP_6 in cell proliferation and differentiation, apoptosis, and potential therapeutic value of IP_6.

REFERENCES

1 **Steinert R**, Buschmann T, van der Linden M, Fels LM, Lippert H, Reymond MA. The role of proteomics in the diagnosis and outcome prediction in colorectal cancer. T*echnol Cancer Res Treat* 2002; **1**: 297-304
2 **Price AS**. Primary and secondary prevention of colorectal cancer. *Gastroenterol Nurs* 2003; **26**: 73-81
3 **Zhang YZ**, Li SY. New technologies of early diagnosis on colorectal cancer. *Shijie Huaren Xiaohua Zazhi* 2004; **12**: 1202-1205
4 **Steinmetz KA**, Kushi LH, Bostick RM, Folsom AR, Potter JD. Vegetables, fruit, and colon cancer in the Iowa Women's Health Study. *Am J Epidemiol* 1994; **139**: 1-15
5 **Greenwald P**, Lanza E, Eddy GA. Dietary fiber in the reduction of colon cancer risk. *J Am Diet Assoc* 1987; **87**: 1178-1188
6 **Helzlsouer KJ**, Block G, Blumberg J, Diplock AT, Levine M, Marnett LJ, Schulplein RJ, Spence JT, Simic MG. Summary of the round table discussion on strategies for cancer prevention: diet, food, additives, supplements, and drugs. *Cancer Res* 1994; **54**: 2044s-2051s
7 **Howe GR**, Benito E, Castelleto R, Cornée J, Estève J, Gallagher RP, Iscovich JM, Deng-ao J, Kaaks R, Kune GA. Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. *J Natl Cancer Inst* 1992; **84**: 1887-1896
8 **Slavin J**, Jacobs D, Marquart L. Whole-grain consumption and chronic disease: protective mechanisms. *Nutr Cancer* 1997; **27**: 14-21
9 **Barbolt TA**, Abraham R. The effect of bran on dimethylhydrazine-induced colon carcinogenesis in the rat. *Proc Soc Exp Biol Med* 1978; **157**: 656-659
10 **Barbolt TA**, Abraham R. Dose-response, sex difference, and the effect of bran in dimethylhydrazine-induced intestinal tumorigenesis in rats. *Toxicol Appl Pharmacol* 1980; **55**: 417-422
11 **Reddy BS**, Mori H. Effect of dietary wheat bran and dehydrated citrus fiber on 3,2'-dimethyl-4-aminobiphenyl-induced intestinal carcinogenesis in F344 rats. *Carcinogenesis* 1981; **2**: 21-25
12 **Alabaster O**, Tang Z, Frost A, Shivapurkar N. Effect of beta-carotene and wheat bran fiber on colonic aberrant crypt and tumor formation in rats exposed to azoxymethane and high dietary fat. *Carcinogenesis* 1995; **16**: 127-132
13 **McIntyre A**, Gibson PR, Young GP. Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. *Gut* 1993; **34**: 386-391
14 **Alabaster O**, Tang ZC, Frost A, Shivapurkar N. Potential synergism between wheat bran and psyllium: enhanced inhibition of colon cancer. *Cancer Lett* 1993; **75**: 53-58
15 **Eastwood MA**. The physiological effect of dietary fiber: an update. *Annu Rev Nutr* 1992; **12**: 19-35
16 **Weisburger JH**, Reddy BS, Rose DP, Cohen LA, Kendall ME, Wynder EL. Protective mechanisms of dietary fibers in nutritional carcinogenesis. *Basic Life Sci* 1993; **61**: 45-63
17 **Velázquez OC**, Lederer HM, Rombeau JL. Butyrate and the colonocyte. Implications for neoplasia. *Dig Dis Sci* 1996; **41**: 727-739
18 **Graf E**, Eaton JW. Dietary suppression of colonic cancer. Fiber or phytate? *Cancer* 1985; **56**: 717-718
19 **Harland BF**, Oberleas D. Phytate in foods. *World Rev Nutr Diet* 1987; **52**: 235-259
20 **Johnson LF**, Tate ME. Structure of "phytic acids". *Can J Chem* 1969; **47**: 63-73
21 **Mills BJ**, Broghamer WL, Higgins PJ, Lindeman RD. Inhibition of tumor growth by zinc depletion of rats. *J Nutr* 1984; **114**: 746-752
22 **Bunce CM**, French PJ, Allen P, Mountford JC, Moor B, Greaves MF, Michell RH, Brown G. Comparison of the levels of inositol metabolites in transformed haemopoietic cells and their normal counterparts. *Biochem J* 1993; **289** (Pt 3): 667-673
23 **Grases F**, Simonet BM, Prieto RM, March JG. Phytate levels in diverse rat tissues: influence of dietary phytate. *Br J Nutr* 2001; **86**: 225-231
24 **Shamsuddin AM**, Vucenik I, Cole KE. IP6: a novel anti-cancer agent. *Life Sci* 1997; **61**: 343-354
25 **Shamsuddin AM**. Inositol phosphates have novel anticancer function. *J Nutr* 1995; **125**: 725S-732S
26 **Warbrick E**. PCNA binding through a conserved motif. *Bioessays* 1998; **20**: 195-199
27 **Lehmann AR**. Dual functions of DNA repair genes: molecular, cellular, and clinical implications. *Bioessays* 1998; **20**: 146-155
28 **Kelman Z**. PCNA: structure, functions and interactions. *Oncogene* 1997; **14**: 629-640
29 **Cayrol C**, Knibiehler M, Ducommun B. p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells.

Oncogene 1998; **16**: 311-320
30 **Aranda FI**, Laforga JB. Cellular proliferation in breast ductal infiltrating carcinoma. Correlation with clinical and histopathological variables. *Pathol Res Pract* 1997; **193**: 683-688
31 **Allred DC**, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998; **11**: 155-168
32 **Cox LS**, Lane DP. Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. *Bioessays* 1995; **17**: 501-508
33 **Lowe SW**, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; **74**: 957-967
34 **Lowe SW**, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T. p53 status and the efficacy of cancer therapy in vivo. *Science* 1994; **266**: 807-810
35 **Culotta E**, Koshland DE Jr. p53 sweeps through cancer research. *Science* 1993; **262**: 1958-1961
36 **Rodrigues NR**, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP. p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990; **87**: 7555-7559
37 **Saied IT**, Shamsuddin AM. Up-regulation of the tumor suppressor gene p53 and WAF1 gene expression by IP_6 in HT-29 human colon carcinoma cell line. *Anticancer Res* 1998; **18**: 1479-1484
38 **Levine AJ**. p53, the cellular gatekeeper for growth and division. *Cell* 1997; **88**: 323-331
39 **Malkowicz SB**, Tomaszewski JE, Linnenbach AJ, Cangiano TA, Maruta Y, McGarvey TW. Novel p21WAF1/CIP1 mutations in superficial and invasive transitional cell carcinomas. *Oncogene* 1996; **13**: 1831-1837
40 **Xiong Y**, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993; **366**: 701-704

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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BASIC RESEARCH

Amplification of chromosome 21q22.3 harboring trefoil factor family genes in liver fluke related cholangiocarcinoma is associated with poor prognosis

Kanuengnuch Muenphon, Temduang Limpaiboon, Patcharee Jearanaikoon, Chawalit Pairojkul, Banchob Sripa, Vajarabhongsa Bhudhisawasdi

Kanuengnuch Muenphon, Temduang Limpaiboon, Patcharee Jearanaikoon, Department of Clinical Chemistry, Center for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
Chawalit Pairojkul, Banchob Sripa, Department of Pathology, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
Vajarabhongsa Bhudhisawasdi, Department of Surgery, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
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Co-first-author: Temduang Limpaiboon
Correspondence to: Dr. Temduang Limpaiboon, Department of Clinical Chemistry, Center for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand. temduang@kku.ac.th
Telephone: +66-43-362028 Fax: +66-43-202088
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Abstract

AIM: To determine allelic imbalance on chromosomal region 21q22-qter including trefoil factor family genes (*TFF*) in cholangiocarcinoma (CCA) patients and analyze the correlation between allelic imbalances and clinicopathological parameters.

METHODS: Quantitative PCR amplification was performed on four microsatellite markers and trefoil factor family genes (*TFF1*, *TFF2*, and *TFF3*) using a standard curve and SYBR Green I dye method. The relative copy number was determined by DNA copy number of tested locus to reference locus. The relative copy number was interpreted as deletion or amplification by comparison with normal reference range. Associations between allelic imbalance and clinicopathological parameters of CCA patients were evaluated by χ^2-tests. Kaplan-Meier method was used to analyze survival.

RESULTS: The frequencies of amplification at D21S1890, D21S1893, and *TFF3* were 32.5%, 30.0%, and 28.7%, respectively. Patients who had amplification at regions covering D21S1893, D21S1890, and *TFF* showed poor prognosis, whereas patients who had deletion showed favorable prognosis (mean: 51.7 wk *vs* 124.82 wk, $P = 0.012$). Multivariate Cox regression analysis revealed that amplification of D21S1893, D21S1890 and *TFF*, blood vessel invasion, and staging were associated with poor prognosis.

CONCLUSION: D21S1893-D21S1890 region may harbor candidate genes especially *TFF* and serine protease family, which might be involved in tumor invasion and metastasis contributing to poor survival. The amplification in this region may be used as a prognostic marker in the treatment of CCA patients.

Key words: Cholangiocarcinoma; Amplification on chromosome 21; Trefoil factor family; Quantitative PCR; Liver fluke

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INTRODUCTION

Cholangiocarcinoma (CCA) or intrahepatic bile duct cancer (ICC) is a malignant tumor in the biliary tree peripheral to the bifurcation of the right and left hepatic duct[1,2]. Incidence rates of ICC vary substantially worldwide, reflecting the distribution of local geographic risk factors, in addition to genetic differences among various populations. In Western countries, the disease is rare, however, it is highly frequent in Southeast Asia, especially in Khon Kaen, Northeast Thailand. Truncated age-standardized incidence of CCA at ages > 35 years varied by three fold between districts, from 93.8 to 317.6 per 100 000 population[3]. In Western countries, primary sclerosing cholangitis is the commonest known predisposing condition for this cancer. Eight percent to 40% of CCA have been reported in patients with primary

Table 1 Locations and sequences of microsatellite markers, trefoil factor family genes and reference loci

Primer name	Chromosome	Product size (bp)	Forward primer	Reverse primer
D21S1253	21q21.3	174-190	GAAGAATCTCCCGAACCAGG	AAGACCAGTGTTATTCAGAGCC
D21S1255	21q22.2	112-126	AGCTCTTTATTTTGCCACATAG	CTGCATGTTTGCCTGG
D21S1893	21q22.2	111-119	GTATGCACACCACACGG	TAACAAAATCCGCCACG
D21S1890	21q22.3	143-173	GGTCTGACCACAGATTTCC	AAAAACACTCTGAACGATTAAGG
Trefoil factor family 1	21q22.3	219	CAGGGATCTGCCTGCATC	ATCGATCTCTTTTAATTTTTAGGCC
Trefoil factor family 2	21q22.3	123	GAAGAATCTCCCGAACCAGG	GTCACACTTCAAAAACTAGAGG
Trefoil factor family 3	21q22.3	129	CAGGCACTGTTCATCTCAGC	TATTCGTTAAGACATCAGGCTCC
β-actin	7p15	375	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG
GAPDH[1]	12p13	250	ACAGTCCATGCCATCACTGCC	GCCTGCTTCACCACCTTCTTG

[1]Glyceraldehyde-3-phosphate dehydrogenase.

sclerosing cholangitis[4]. Several clinical studies and animal model experimental studies suggest that the interaction between chemical carcinogens, especially nitrosamines and *Opisthorcis viverrini* infestation may play an important role in the development of CCA in Thailand[5-7]. Thus, either the chemical carcinogen nitrosamine or liver fluke infection alone does not produce cancer. Food derived exogenous and *in situ* nitrosamine formation may lead to DNA alkylation and also deamination in predisposed and inflamed tissues. Furthermore, chronic irritation caused by the fluke results in hyperplasia and adenomatous change of bile duct epithelium[6]. The DNA damaged biliary epithelium may then be transformed to malignant CCA[7-9]. To date, the molecular basis of carcinogenesis and pathogenesis of cholangiocarcinoma is still unclear.

Allelic imbalance at specific genomic loci is an important step in the molecular genetic analysis of human cancers. Allelic imbalance at chromosome 21, especially region 21q22-qter, was found in several types of human cancers such as gastric cancer, breast cancer, ovarian clear cell adenocarcinoma, and primary colorectal cancer[10-13]. Furthermore, chromosome 21q22.3 harbors a cluster of trefoil factor family (*TFF*) genes consisting of *TFF1*, *TFF2*, and *TFF3*[14]. TFF functions include mucus stabilization and stimulation of normal epithelial cell restitution during wound repair through motogenic and antiapoptotic activities. However, TFF peptides are overexpressed in several human solid tumors such as prostate, esophagus, breast, and pancreas and also function as tumor progression factor[15-18]. Prolonged inflammation caused by parasitic infection frequently occurs in liver fluke related CCA. *TFF* and its neighborhood located at 21q22 may be involved in tumor development and progression. Moreover, our data on comparative genomic hybridization (CGH) in CCA showed the alteration of DNA copy number at 21q22-qter at 28%.

Taken these data together, the chromosomal region 21q22-qter may harbor candidate genes, which are involved in carcinogenesis and pathogenesis of CCA. Therefore, this study attempted to determine allelic imbalance on chromosomal region 21q22-qter including *TFF* genes to define affected sites for candidate genes which are involved in molecular carcinogenesis and pathogenesis of CCA. The associations between allelic imbalance and clinicopathological parameters were also determined.

MATERIALS AND METHODS

Samples and DNA preparation

This project was approved by the Ethical Committee of Khon Kaen University. Informed consents were obtained from patients who were willing to participate in the project. Frozen liver tissues were obtained from 80 CCA patients undergoing surgical resection at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. CCA cases were diagnosed by physicians according to clinical finding, laboratory investigation and histological examination. Neural, blood vessel and lymphatic invasion were assessed by standard method[19]. The clinicopathological data such as age, gender, histological type, and TNM stage[20] were evaluated by reviewing the medical charts and pathology records. DNA was prepared from frozen liver tissues containing 80% of tumor cells by using a Puregene™ DNA purification system (Gentra System, USA) according to manufacturer's instructions. In addition, DNA was prepared from placental tissue collected from a normal labor (postpartum) woman and used for setting a standard curve. Normal leukocyte DNA derived from 50 healthy donors was prepared into 14 pooled normal DNA and generated for normal reference range.

Quantitative PCR assay

Quantitative PCR amplification was performed on a Rotor Gene 2000 Real-time Amplification (Corbett Research, Australia) using four microsatellite markers (telomere-D21S1890-D21S1893-D21S1255-D21S1253-centromere) and trefoil factor family genes (*TFF1*, *TFF2*, and *TFF3*) covering chromosomal region 21q21-qter. Reference primers were chosen in the region of the housekeeping genes that usually are not altered alteration in CCA, β-*actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences were obtained from the Genome Data Base. Locations of selected oligonucleotides and their sequences are shown in Table 1. PCR reaction was performed in a 25 μL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.3, 100 μmol/L each of deoxynucleoside triphosphate (dNTP), 2.5 mmol/L $MgCl_2$ or 3.0 mmol/L (D21S1253, *TFF1*, and β-*actin*), 1.25 μL SYBR® Green I dye solution (Amresco, USA) (1:10 000 in DMSO), and 10 μmol/L or 5 μmol/L (D21S1253 and

D21S1255) or 15 μmol/L (D21S1890) of each primer with 1.5 units or 2.0 units (D21S1253, D21S1255, and *GAPDH*) of *Taq* DNA polymerase. The PCR was performed at 95℃ for 5 min followed by 95℃ for 15 s, 45℃ -60℃ for 15 s and 72℃ for 15 s for 35 cycles with an additional cycle of 72℃ for 10 min.

Quantitative PCR amplification was performed using a standard curve and SYBR Green I dye method as described previously[21]. The standard curve for each primer was generated using serial dilutions of placental DNA. The standard curve was constructed in each PCR run and the copy numbers of genes in each sample were interpolated using these standard curves. Placental DNA with known concentration was used for precision control. A coefficient of variation (CV) of each sample was determined based on triplicate test. The sample with a CV higher than 15% was re-tested. DNA copy number of each locus was calculated based on triplicate determination and duplicate PCR run.

Analysis of allelic imbalance

The relative copy number was determined by DNA copy number of tested locus to DNA copy number of reference locus. The DNA copy numbers of reference loci consisting of β-*actin* and *GAPDH* were averaged before calculation. The normal reference range was generated from the relative copy numbers of 14 pooled normal leukocyte DNA of 7 markers (n = 98). If the relative copy number of sample calculated differed significantly from normal reference range (mean ± 2SD), the sample was verified as loss or gain. The relative copy number was interpreted as loss when the ratio was less than mean -2SD of normal reference range. On the other hand, the relative copy number was interpreted as gain when the ratio was more than mean + 2SD of normal reference range.

Statistical analysis

Associations between allelic imbalance and clinicopathological parameters of 80 CCA patients were evaluated by means of the χ^2-tests. Survival curves for patients with allelic imbalance versus those without were calculated using the Kaplan-Meier method. Only 69 cases were available for follow-up. Six patients were lost for follow-up and five cases were perioperative death (patients who died within 4 wk after surgery). Differences in survival between these two groups were assessed by the log-rank test. Cox proportional hazards model was used in univariate and multivariate analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Allelic imbalance on chromosomal region 21q22-qter

The normal reference range generated from relative copy numbers of pooled normal leukocyte DNA was 0.54-1.34 with 95% confidence interval (mean ± 2SD). Allelic imbalance of 7 loci showed percentages of amplification at D21S1890 (32.5%), D21S1893 (30.0%), *TFF3* (28.8%), D21S1253 (26.3%), D21S1255 (23.8%), *TFF1* (22.5%), and *TFF2* (7.5%) and of deletion at *TFF3* (3.8%), D21S1255 (2.5%), *TFF2* (2.5%), and D21S1890 (1.3%).

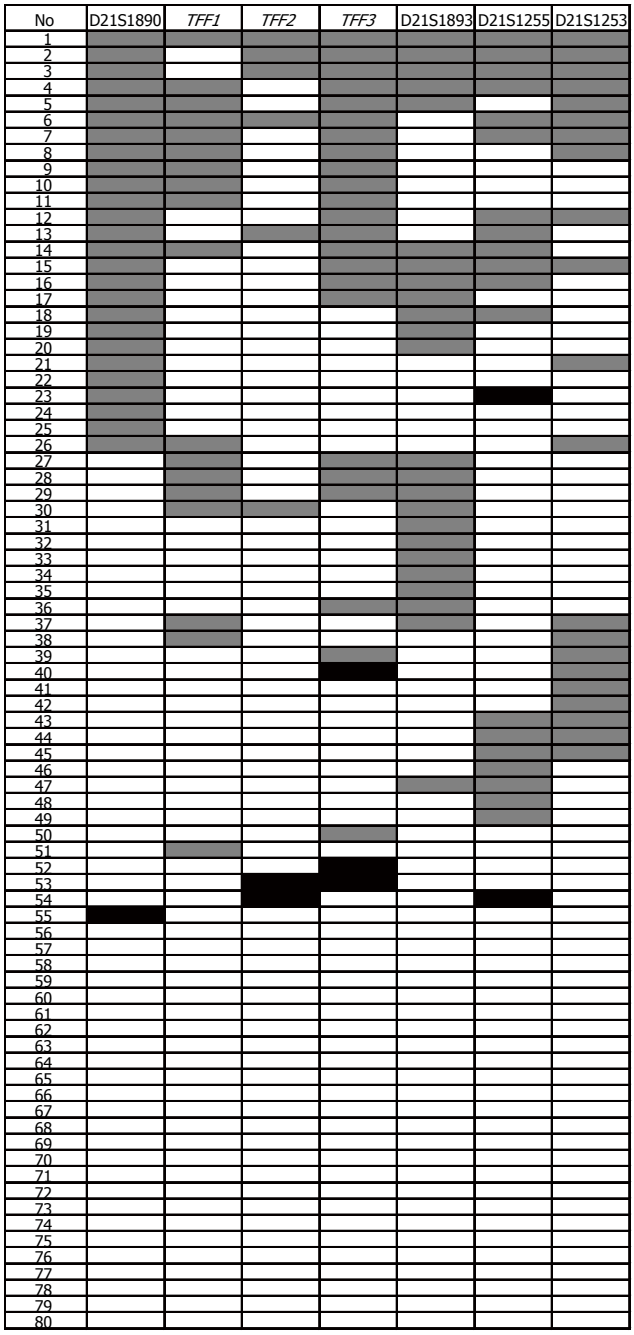

Figure 1 Fine mapping of allelic imbalance on chromosomal region 21q22-qter. D21S1890 is located at telomeric end while D21S1253 is located toward centromeric end. There are two common amplification regions at D21S1890 and the region between D21S1893 and *TFF*3.

The relative copy number of amplification of these loci ranged between 1.35-4.24 and of deletion between 0.39-0.52. Fine mapping of these regions is shown in Figure 1. Two regions of common amplification were D21S1890 and the region between D21S1893 and *TFF3*.

Associations between allelic imbalance and clinicopathological parameters of patients

The associations between allelic imbalance and clinicopathological parameters of patients were analyzed. The result showed no differences in age, sex, histological type, invasion (blood vessel, lymphatic, and nerve), and survival time between patients with and without

Table 2 Clinicopathological parameters of patients with and without allelic imbalance

Clinical parameters	*n*	Gene/microsatellite makers D21S1893, D21S1890 and *TFF*			
		Normal *n* (%)	Amplification *n* (%)	Deletionl *n* (%)	*P* value
Age (yr)	80				
⩽ 54	39	18 (46)	20 (51)	1 (3)	NS
> 54	41	15 (36)	22 (54)	4 (10)	
Gender	80				
Male	57	22 (39)	32 (56)	3 (5)	NS
Famale	23	11 (48)	10 (43)	2 (9)	
Histological type	80				
Non-Papillary adenocarcinoma	58	27 (46)	30 (52)	1 (2)	0.023
Papillary adenocarcinoma	22	6 (27)	12 (55)	4 (18)	
Staging	80				
II & III	10	5 (50)	5 (50)	-	NS
IVA & IVB	70	28 (40)	37 (52.8)	5 (7.2)	
Blood vessel invasion	79				
Non-invasion	28	8 (29)	16 (57)	4 (14)	NS
Invasion	51	24 (47)	26 (51)	1 (2)	
Lymphatic invasion	79				
Non-invasion	15	4 (27)	11 (73)	-	NS
Invasion	64	28 (43)	31 (49)	5 (8)	
Nerve invasion	79				
Non-invasion	36	14 (39)	19 (53)	3 (8)	NS
Invasion	43	18 (42)	23 (54)	2 (4)	
Survival time (wk)	69				0.012
Mean		54.12	51.7	124.82	
Median		49	27.4	84.28	
Minimum-maximum		7.14-119.28	4.85-242.85	67.71-143.85	

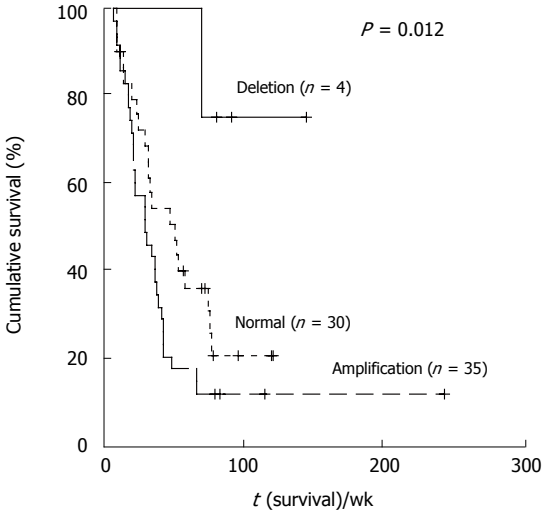

Figure 2 The association between survival time and allelic imbalance on loci D21S1893, D21S1890 and TFF was analyzed by Kaplan-Meier. The patients who had amplification at D21S1893, D21S1890 and TFF showed poor prognosis, whereas patients who had deletion at D21S1893, D21S1890 and TFF showed favorable prognosis.

allelic imbalance in almost all loci ($P > 0.050$). However, significant difference was observed in histological type at loci covering D21S1893 through D21S1890 including *TFF* ($P = 0.023$). Deletion at D21S1893, D21S1890, and *TFF* found in a papillary type was higher than that in a non-papillary one (Table 2). Furthermore, patients who had deletion in this region tended to show non-invasion of blood vessel ($P = 0.052$) (Table 2). Kaplan-Meier survival curves demonstrated that patients who had amplification at D21S1893, D21S1890, and *TFF* showed poor prognosis, whereas patients who had deletion at D21S1893, D21S1890, and *TFF* showed favorable prognosis ($P = 0.012$) (Figure 2). Multivariate analysis showed significant effects of amplification at D21S1893, D21S1890, and *TFF*, blood vessel invasion, and staging on prognosis (Table 3).

DISCUSSION

Many techniques have been used to detect genetic alterations. The real-time qPCR technique is an alternative method to determine allelic imbalance because most allelic imbalances also result in changes of relative copy numbers. In our study, allelic imbalance determined by qPCR using SYBR Green I system showed CV less than 15%, suggesting good consistency and reliability[21]. The house keeping genes, β-*actin* and *GAPDH,* were used as reference loci in our study. To assess the validity of these reference genes, we determined the copy number ratios of β-*actin*/*GAPDH* in 14 pooled normal leukocytes and 80 CCA samples. The observed ratios measured from normal DNA (1.01 ± 0.14SD) and tumor DNA (1.06 ± 0.15SD) were similar ($P = 0.271$) and both were significantly equal to 1, thus, confirming their validity as appropriate reference genes in our work.

Our finding of allelic imbalance on chromosomal region 21q22-qter in CCA patients showed predominant amplifications at markers D21S1890 (32.5%) and D21S1893 (30%). This region has length about 3.5 Mb in physical map distance and contains about 25 identified genes. Trefoil factor family (*TFF1, TFF2*, and *TFF3*) and serine protease family (*TMPRSS2* and *TMPRSS3)* are candidate genes located in this region and have a potential tumor progression activity. In this study, all CCA tissues were obtained from patients who were residents of northeastern region of Thailand where liver fluke infection remains highly endemic. Increased gene amplification at chromosome 21q22.3 especially *TFF* genes in liver fluke related CCA may result from healing process of inflamed tissues. Normally, TFF peptides are involved in the normal mucosal defense and epithelial restitution in cell injury. However, in chronic inflammation, the overproduction of TFF peptides may result in tumor progression. TFF peptides might exert beneficial effects during the early step of mucosal injury and inflammation and subsequently undesirable effects during chronic inflammation and neoplastic progression. TFF peptides function as a tumor progression factor by increasing cell scattering, invasion, survival and proangiogenesis[22-25]. TFF3 stimulates cell motility by inducing a rapid phosphorylation of β-catenin, which is associated with perturbation of the functional integrity of E-cadherin/catenin system. The promotion of cell motility in association with epidermal growth factor receptor (EGFR) leads to the enhancement of tumor cell invasion and metastasis[26,27]. However, exogenous TFF peptides

Table 3 Univariate and multivariate analysis of overall survival in cholangiocarcinoma

Variable	*n*	Univariate		Multivariate	
		Relative risk (95%CI)	*P* value	Relative risk (95%CI)	*P* value
Age (yr)	69	0.831 (0.483-1.430)	NS		
≤ 54	34				
> 54	35				
Gender	69	0.443 (0.231-0.849)	0.014	0.628 (0.312-1.263)	NS
Male	49				
Famale	20				
Histological type	69	0.622 (0.332-1.167)	NS		
Non-Papillary adenocarcinoma	50				
Papillary adenocarcinoma	19				
Staging	69	2.265 (0.899-5.708)	0.083	3.320 (1.270-8.681)	0.014
II & III	9				
IVA & IVB	60				
Blood vessel invasion	69	2.108 (1.139-3.902)	0.018	2.183 (1.088-4.382)	0.028
Non-invasion	23				
Invasion	46				
Lymphatic invasion	69	0.981 (0.479-2.012)	NS		
Non-invasion	12				
Invasion	57				
Nerve invasion	69	1.129 (0.654-1.950)	NS		
Non-invasion	32				
Invasion	37				
D21S1893, D21S1890, and TFF	69		0.026		0.002
Normal	30	Reference		Reference	
Amplification	35	1.707 (0.974-2.991)	NS	2.473 (1.342-4.557)	0.004
Deletion	4	0.187 (0.025-1.394)	NS	0.224 (0.029-1.701)	NS

alone are not sufficient to induce the invasive phenotype in premalignant human colonic adenoma cells PC/AA/C1 and kidney MDCK epithelial cells, but require the priming and permissive action of src and RhoA to exert their proinvasive activity[22]. These observations suggest that trefoil peptides elicit a coordinated cellular response enabling cell migration without triggering the programmed cell death response usually precipitated by cell detachment from a stationary anchored state. TFF1 protects cells from anoikis, chemical-, or Bad-induced apoptosis by reducing caspase-3, caspase-6, caspase-8, and caspase-9 activities[25]. TFF3 may act as anti-apoptosis by preventing p53-dependent and p53-independent apoptosis pathways[28]. The anti-apoptotic effects of TFF3 are associated with activation of the PI3K-Akt signaling pathway. Thus, TFF functions as an anti-apoptosis factor, resulting in an increase in number of survived cancer cells through proliferation *via* Ras/MEK/MAP kinase signaling transduction pathway. Angiogenic activity of TFF is comparable to that induced by vascular endothelial growth factor (VEGF), leptin, and transforming growth factor-alpha. Stimulation of angiogenesis by TFF1 in the chick chorioallantoic membrane (CAM) assay was COX-2- and EGFR-dependent, but independent of the VEGF receptor KDR/flk-1 and the thromboxane A2 receptor (TXA-2-R). These results implicate a role of TFF in the formation of new blood vessels during normal and pathophysiological processes linked to wound healing, inflammation, and cancer progression[23].

Transmembrane protease, serine 2 *(TMPRSS2)* and transmembrane protease, serine 3 (*TMPRSS3*) are members of serine protease family. Proteases have been increasingly recognized as important factors in the pathophysiology of tumorous diseases. Members of the endopeptidases, such as serine protease family, mediate the proteolytic degradation of the extracellular matrix, which is an indispensable step in tumor invasion and metastasis[29]. *TMPRSS2* was highly expressed in prostate cancer and correlated with the metastatic potential and involved in microvascular endothelial cell reorganization and capillary morphogenesis[30,31]. *TMPRSS3* is strongly expressed in a subset of pancreatic cancer and various other cancer tissues, and its expression correlates with the metastatic potential of the clonal SUIT-2 pancreatic cancer cell lines[32]. The data suggested that both *TMPRSS2* and *TMPRSS3* may be important for the processes involved in metastasis, invasion, and angiogenesis in tumor cells. Our study showed high amplifications of markers D21S1893 and D21S1890, suggesting that existence of candidate genes might be involved in pathogenesis of CCA. The data regarding the involvement of serine protease and *TFF* in aggressive feature and metastasis supported our finding that amplifications of candidate genes at regions D21S1893, D21S1890, and *TFF* were found in poor prognostic CCA patients. Clinical data of CCA patients supported our hypothesis that TFF stimulated cell motility via E-cadherin/catenin and APC complexes and promoted tumor cell survival by anti-apoptosis, while serine protease mediated vascular endothelial invasion and angiogenesis leading to poor survival in CCA patients. Although the differences in genetic alterations between liver fluke related and non-liver fluke related CCA have been observed previously[33,34], the conclusion regarding the difference between these two groups in allelic imbalance on chromosome 21q22 cannot be drawn.

As far as we know, allelic imbalance on the chromosomal region 21q22-qter including *TFF* in CCA patients is first reported by our group. The protein expression and functions of *TMPRSS2, TMPRSS3,* and *TFF* related to cancer invasion and metastasis in liver fluke related CCA patients require further study. The application of allelic imbalance on D21S1893, D21S1890, and *TFF* may be of value as a prognostic marker and a selection for CCA patient treatment.

REFERENCES

1 **Uttaravichien T**, Bhudhisawasdi V, Pairojkul C, Pugkhem A. Intrahepatic cholangiocarcinoma in Thailand. *J Hepatobiliary Pancreat Surg* 1999; **6**: 128-135

2 **Nakanuma Y**, Hoso M, Sanzen T, Sasaki M. Microstructure and development of the normal and pathologic biliary tract in humans, including blood supply. *Microsc Res Tech* 1997; **38**: 552-570

3 **Sriamporn S**, Pisani P, Pipitgool V, Suwanrungruang K, Kamsa-ard S, Parkin DM. Prevalence of Opisthorchis viverrini infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. *Trop Med Int Health* 2004; **9**: 588-594

4 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125
5 **Watanapa P**, Watanapa WB. Liver fluke-associated cholangiocarcinoma. *Br J Surg* 2002; **89**: 962-970
6 **Bhamarapravati N**, Thammavit W, Vajrasthira S. Liver changes in hamsters infected with a liver fluke of man, Opisthorchis viverrini. *Am J Trop Med Hyg* 1978; **27**: 787-794
7 **Thamavit W**, Kongkanuntn R, Tiwawech D, Moore MA. Level of Opisthorchis infestation and carcinogen dose-dependence of cholangiocarcinoma induction in Syrian golden hamsters. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1987; **54**: 52-58
8 **Thamavit W**, Moore MA, Hiasa Y, Ito N. Enhancement of DHPN induced hepatocellular, cholangiocellular and pancreatic carcinogenesis by Opisthorchis viverrini infestation in Syrian golden hamsters. *Carcinogenesis* 1988; **9**: 1095-1098
9 **Thamavit W**, Pairojkul C, Tiwawech D, Shirai T, Ito N. Strong promoting effect of Opisthorchis viverrini infection on dimethylnitrosamine-initiated hamster liver. *Cancer Lett* 1994; **78**: 121-125
10 **Ohgaki K**, Iida A, Kasumi F, Sakamoto G, Akimoto M, Nakamura Y, Emi M. Mapping of a new target region of allelic loss to a 6-cM interval at 21q21 in primary breast cancers. *Genes Chromosomes Cancer* 1998; **23**: 244-247
11 **Suehiro Y**, Sakamoto M, Umayahara K, Iwabuchi H, Sakamoto H, Tanaka N, Takeshima N, Yamauchi K, Hasumi K, Akiya T, Sakunaga H, Muroya T, Numa F, Kato H, Tenjin Y, Sugishita T. Genetic aberrations detected by comparative genomic hybridization in ovarian clear cell adenocarcinomas. *Oncology* 2000; **59**: 50-56
12 **Park WS**, Oh RR, Park JY, Yoo NJ, Lee SH, Shin MS, Kim SY, Kim YS, Lee JH, Kim HS, An WG, Lee JY. Mapping of a new target region of allelic loss at 21q22 in primary gastric cancers. *Cancer Lett* 2000; **159**: 15-21
13 **Aragane H**, Sakakura C, Nakanishi M, Yasuoka R, Fujita Y, Taniguchi H, Hagiwara A, Yamaguchi T, Abe T, Inazawa J, Yamagishi H. Chromosomal aberrations in colorectal cancers and liver metastases analyzed by comparative genomic hybridization. *Int J Cancer* 2001; **94**: 623-629
14 **Thim L**. A new family of growth factor-like peptides. 'Trefoil' disulphide loop structures as a common feature in breast cancer associated peptide (pS2), pancreatic spasmolytic polypeptide (PSP), and frog skin peptides (spasmolysins). *FEBS Lett* 1989; **250**: 85-90
15 **Colombel M**, Dante R, Bouvier R, Ribieras S, Pangaud C, Marechal JM, Lasne Y. Differential RNA expression of the pS2 gene in the human benign and malignant prostatic tissue. *J Urol* 1999; **162**: 927-930
16 **Labouvie C**, Machado JC, Carneiro F, Sarbia M, Vieth M, Porschen R, Seitz G, Blin N. Differential expression of mucins and trefoil peptides in native epithelium, Barrett's metaplasia and squamous cell carcinoma of the oesophagus. *J Cancer Res Clin Oncol* 1999; **125**: 71-76
17 **May FE**, Westley BR. Expression of human intestinal trefoil factor in malignant cells and its regulation by oestrogen in breast cancer cells. *J Pathol* 1997; **182**: 404-413
18 **Terris B**, Blaveri E, Crnogorac-Jurcevic T, Jones M, Missiaglia E, Ruszniewski P, Sauvanet A, Lemoine NR. Characterization of gene expression profiles in intraductal papillary-mucinous tumors of the pancreas. *Am J Pathol* 2002; **160**: 1745-1754
19 **Nakanuma Y**, Harada K, Ishikawa A, Zen Y, Sasaki M. Anatomic and molecular pathology of intrahepatic cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2003; **10**: 265-281
20 **Tio TL**, Wijers OB, Sars PR, Tytgat GN. Preoperative TNM classification of proximal extrahepatic bile duct carcinoma by endosonography. *Semin Liver Dis* 1990; **10**: 114-120
21 **De Preter K**, Speleman F, Combaret V, Lunec J, Laureys G, Eussen BH, Francotte N, Board J, Pearson AD, De Paepe A, Van Roy N, Vandesompele J. Quantification of MYCN, DDX1, and NAG gene copy number in neuroblastoma using a real-time quantitative PCR assay. *Mod Pathol* 2002; **15**: 159-166
22 **Emami S**, Le Floch N, Bruyneel E, Thim L, May F, Westley B, Rio M, Mareel M, Gespach C. Induction of scattering and cellular invasion by trefoil peptides in src- and RhoA-transformed kidney and colonic epithelial cells. *FASEB J* 2001; **15**: 351-361
23 **Rodrigues S**, Nguyen QD, Faivre S, Bruyneel E, Thim L, Westley B, May F, Flatau G, Mareel M, Gespach C, Emami S. Activation of cellular invasion by trefoil peptides and src is mediated by cyclooxygenase- and thromboxane A2 receptor-dependent signaling pathways. *FASEB J* 2001; **15**: 1517-1528
24 **Prest SJ**, May FE, Westley BR. The estrogen-regulated protein, TFF1, stimulates migration of human breast cancer cells. *FASEB J* 2002; **16**: 592-594
25 **Bossenmeyer-Pourié C**, Kannan R, Ribieras S, Wendling C, Stoll I, Thim L, Tomasetto C, Rio MC. The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. *J Cell Biol* 2002; **157**: 761-770
26 **Liu D**, el-Hariry I, Karayiannakis AJ, Wilding J, Chinery R, Kmiot W, McCrea PD, Gullick WJ, Pignatelli M. Phosphorylation of beta-catenin and epidermal growth factor receptor by intestinal trefoil factor. *Lab Invest* 1997; **77**: 557-563
27 **Efstathiou JA**, Noda M, Rowan A, Dixon C, Chinery R, Jawhari A, Hattori T, Wright NA, Bodmer WF, Pignatelli M. Intestinal trefoil factor controls the expression of the adenomatous polyposis coli-catenin and the E-cadherin-catenin complexes in human colon carcinoma cells. *Proc Natl Acad Sci USA* 1998; **95**: 3122-3127
28 **Kinoshita K**, Taupin DR, Itoh H, Podolsky DK. Distinct pathways of cell migration and antiapoptotic response to epithelial injury: structure-function analysis of human intestinal trefoil factor. *Mol Cell Biol* 2000; **20**: 4680-4690
29 **DeClerck YA**, Imren S. Protease inhibitors: role and potential therapeutic use in human cancer. *Eur J Cancer* 1994; **30A**: 2170-2180
30 **Aimes RT**, Zijlstra A, Hooper JD, Ogbourne SM, Sit ML, Fuchs S, Gotley DC, Quigley JP, Antalis TM. Endothelial cell serine proteases expressed during vascular morphogenesis and angiogenesis. *Thromb Haemost* 2003; **89**: 561-572
31 **Vaarala MH**, Porvari K, Kyllönen A, Lukkarinen O, Vihko P. The TMPRSS2 gene encoding transmembrane serine protease is overexpressed in a majority of prostate cancer patients: detection of mutated TMPRSS2 form in a case of aggressive disease. *Int J Cancer* 2001; **94**: 705-710
32 **Wallrapp C**, Hähnel S, Müller-Pillasch F, Burghardt B, Iwamura T, Ruthenbürger M, Lerch MM, Adler G, Gress TM. A novel transmembrane serine protease (TMPRSS3) overexpressed in pancreatic cancer. *Cancer Res* 2000; **60**: 2602-2606
33 **Kiba T**, Tsuda H, Pairojkul C, Inoue S, Sugimura T, Hirohashi S. Mutations of the p53 tumor suppressor gene and the ras gene family in intrahepatic cholangiocellular carcinomas in Japan and Thailand. *Mol Carcinog* 1993; **8**: 312-318
34 **Limpaiboon T**, Krissadarak K, Sripa B, Jearanaikoon P, Bhuhisawasdi V, Chau-in S, Romphruk A, Pairojkul C. Microsatellite alterations in liver fluke related cholangiocarcinoma are associated with poor prognosis. *Cancer Lett* 2002; **181**: 215-222

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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BASIC RESEARCH

Antioxidant role of heme oxygenase-1 in prehepatic portal hypertensive rats

Soledad Gonzales, María Julia Pérez, Juan C Perazzo, María Luján Tomaro

Soledad Gonzales, María Luján Tomaro, Department of Biological Chemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires 1113, Argentina
María Julia Pérez, Juan C Perazzo, Laboratory of Portal Hypertension, Department of Biological Science, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires 1113, Argentina
Co-first-authors: María Julia Pérez and Juan C Perazzo
Supported by Grants from the University of Buenos Aires, Buenos Aires, Argentina and CONICET, Buenos Aires, Argentina
Correspondence to: Professor Juan C Perazzo, Laboratory of Portal Hypertension, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, CP 1113, Ciudad Autónoma de Buenos Aires,
Republica Argentina. jperazzo@ffyb.uba.ar
Telephone: +54-11-49648368 Fax: +54-11-49648274
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Abstract

AIM: To study the effect of bilirubin on the oxidative liver status and the activity and expression of heme oxygenase-1 (HO-1) in rat liver injury induced by prehepatic portal hypertension.

METHODS: Wistar male rats, weighing 200-250 g, were divided at random into two groups: one group with prehepatic portal hypertension (PH) induced by regulated prehepatic portal vein ligation (PPVL) and the other group corresponded to sham operated rats. Portal pressure, oxidative stress parameters, antioxidant enzymes, HO-1 activity and expression and hepatic sinusoidal vasodilatation were measured.

RESULTS: In PPVL rats oxidative stress was evidenced by a marked increase in thiobarbituric acid reactive substances (TBARS) content and a decrease in reduced glutathione (GSH) levels. The activities of liver antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were also diminished while activity and expression of HO-1 were enhanced. Administration of bilirubin (5 μmol/kg body weight) 24 h before the end of the experiment entirely prevented all these effects. Pretreatment with Sn-protoporphyrin IX (Sn-PPIX) (100 μg/kg body weight, i.p.), a potent inhibitor of HO, completely abolished the oxidative stress and provoked a slight decrease in liver GSH levels as well as an increase in lipid peroxidation. Besides, carbon monoxide, another heme catabolic product, induced a significant increase in sinusoidal hepatic areas in PPVL group. Pretreatment of PPVL rats with Sn-PPIX totally prevented this effect.

CONCLUSION: These results suggest a beneficial role of HO-1 overexpression in prehepatic portal hypertensive rats.

Key words: Heme oxygenase-1; Portal hypertensive rats; Liver oxidative stress

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INTRODUCTION

Portal hypertension (PH) constitutes a major complication in chronic liver diseases including cirrhosis. It is associated with a hyperdynamic splanchnic circulation in response to the resistance of portal blood flow to reach the liver. This complication is associated with collateralization of the portal system, leading to the development of varix at various locations in the upper gastrointestinal tract[1].

Oxidative stress is the result of excessive generation of reactive oxygen species (ROS), depletion of intracellular antioxidant defences or a combination of both, leading to an imbalance in the redox status of the cell. Reactive oxygen species induce cell, tissue or organ damage and ROS have been proposed as a major factor responsible for several diseases, and have been implicated in portal hypertension[2].

ROS occur in tissues and may damage DNA, proteins, carbohydrates, and lipids. These potentially deleterious reactions are controlled by a system of antioxidant defences, which eliminate pro-oxidants and scavenge free radicals. Various intracellular compounds such as glutathione, and antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px)[3] provide protection against oxidation. Heme oxygenase (HO) is the key microsomal enzyme in heme degradation to carbon monoxide (CO), iron and biliverdin, the latter being converted into bilirubin by the cytosolic biliverdin reductase[4,5]. Heme oxygenase, the rate limiting enzyme in heme degradation pathway, is induced in animal tissues, particularly in liver by many factors including its own

substrate heme, several heme-proteins, heavy metals, UVA radiation, hypoxia, hyperoxia and others[6-9]. Induction of HO is entirely prevented by administration of several antioxidants such as α-tocopherol and allopurinol[9]. In recent years good evidence has been accumulated showing that bilirubin can act as a highly effective antioxidant and free radical scavenger, and it has been proposed that it can play a physiological and key role as an endogenous protective agent against oxidant mediated injury[10-14].

Antioxidant effects suggest that oxidant species play a major part in the induction of HO either directly or by GSH depletion[9,10,15]. An increase in HO activity will enhance bilirubin formation and because unconjugated bilirubin is an efficient scavenger of ROS, its increase would be the cellular response to oxidative stress[10,12-14]. Besides, similar to nitric oxide, CO derived from heme oxygenase reaction also acts as a neurotransmitter and regulator of vascular tone[16].

Of the two known mammal liver HO isoenzymes, HO-1 and HO-2[17], only HO-1 is inducible, herewith we will only refer to HO-1.

The aim of this work was to investigate the effect of HO-1 overexpression in rat livers with prehepatic portal hypertension.

MATERIALS AND METHODS

Animals and surgical procedures

Wistar male rats (200-250 g) were housed separately and acclimatised before use under conditions of controlled temperature (25 ± 2℃) and illumination (12 h light/dark cycle). Rats were fed with standard rat chow and water *ad libitum*. After 1 wk of acclimatisation, rats were randomised and separated into two groups: (1) Sham operated (n = 24) and (2) Prehepatic portal vein ligated rats (n = 24) (PPVL). Portal hypertension was induced by a calibrated portal vein stenosis according to the procedure of Chojkier *et al*[18]. In brief, rats were anaesthetised with sodium pentobarbital (50 mg/kg body weight, i.p.) and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3-0 silk was placed around the vein and snugly tied it to a 20 gauge blunt end needle placed along side the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Sham rats underwent an identical procedure except that portal vein was isolated but not stenosed. Fourteen days after the operation rats of group 2 developed portal hypertension. Then, one day before the experiment, portal hypertensive and Sham rats were divided into three subgroups. One of each subgroup received one bolus injection of bilirubin (5 μmol/kg body weight, i.p) or one bolus injection of Sn-protoporphyrin IX (Sn-PPIX; 100 μmol/kg body weight, i.p). Then, six groups of animals were used (n = 8): Sham group (Sham), PPVL, Sham pretreated with bilirubin group (Sham + bilirubin), PPVL pretreated with bilirubin group (PPVL + bilirubin), sham pretreated with Sn-PPIX group (Sham + Sn-PPIX) and PPVL pretreated with Sn-PPIX group (PPVL + Sn-PPIX). Bilirubin and Sn-PPIX were prepared as follows: solutions in 0.1 mol/L NaOH were freshly prepared before administration, adjusted to pH 7.4 with phosphate buffer and diluted with saline. In each subgroup portal pressure was measured immediately before the sacrifice. Fourteen days after the corresponding operation and before sacrifice, the rats were anaesthetised with sodium pentobarbital (50 mg/kg), intraperitoneally (i.p.). Portal pressure was measured through a needle placed in the splenic pulp, and maintained in place by cyanoacrylate gel. The needle was cannulated to a polyethylene catheter (50) filled with a heparinized saline solution (25 U/mL) and connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico) coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA). Animals were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC), and were in accordance with the Guide to the Care and Use of Experimental Animals published by the Argentine Council on Animal Care.

Enzyme preparations and assays

Rats were anaesthetised with sodium pentobarbital (50 mg/kg body weight, i.p.) and killed 14 d after surgery. Livers were excised and perfused "in situ" with ice-cold saline solution (0.9% NaCl), then excised and homogenised in a Potter-Elvehejm homogenizer using different solutions. For heme oxygenase assay the homogenate was prepared using 4 vol of ice-cold 0.25 mol/L sucrose solution containing 1 mmol/L phenylmethyl sulfonyl fluoride, 0.2 mmol/L EDTA and 50 mmol/L potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20 000 g for 20 min and supernatant fractions centrifuged at 150 000 g for 90 min. The microsomal pellet obtained was washed and resuspended in 20 mmol/L potassium phosphate buffer (pH 7.4), containing 135 mmol/L KCl, 1 mmol/L phenyl-methylsulfonyl fluoride and 0.2 mmol/L EDTA to a protein concentration of 10 mg/mL. Microsomal HO-1 was obtained as described elsewhere[17]. The 150 000 g supernatants obtained from the microsomal preparation were fractionated by addition of ammonium sulfate (AS), and the 40%-60% AS fraction dissolved in 10 mmol potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer using this preparation as biliverdin reductase. Heme oxygenase activity was determined as described elsewhere[10]. The standard incubation mixture in a final volume of 200 μL contained 10 μmol potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 50 μL HO-1 (0.5 mg protein), 50 μL biliverdin reductase (0.42 mg protein), and 200 nmol hemin. Incubations were carried out at 37℃ for 30 min. Activity was determined by measuring bilirubin formation, which was calculated as the difference in absorbance measured at 455 and 520 nm, employing an ε value of 50 mM^{-1} cm^{-1} (vis_{max} 455 nm)[11]. CAT, SOD and GSH-Px activities were determined spectrophotometrically in liver homogenates prepared in a medium consisting of 140 mmol/L KCl and 25 mmol/L potassium phosphate buffer (pH 7.4), and centrifuged at 600 g for 10 min. The supernatant, a suspension of preserved organelles, was used as homogenate. Catalase activity was determined by measuring the decrease in absorbance at 240 nm[19] glutathione peroxidase activity following NADPH oxidation at 340 nm[20], and superoxide dismutase

activity by inhibition of adrenochrome formation rate at 480 nm[21]. One unit in the SOD assay is defined as the amount of enzymatic protein required to inhibit 50% of epinephrine auto-oxidation.

Lipid peroxidation

Lipid peroxidation in liver was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde equivalents[22]. One volume of homogenate was mixed with 0.5 volume TCA (15% w/v) and centrifuged at 2000 g for 10 min. The supernatant (1 mL) was mixed with 0.5 mL thiobarbituric acid (0.7% w/v) and boiled for 10 min. After cooling, sample absorbance was read spectrophotometrically at 535 nm. Malondialdehyde concentration was calculated using an ε value of 1.56×10^5 $M^{-1}cm^{-1}$.

Endogenous hepatic GSH content

Total glutathione (GSH plus GSSG) was determined in liver homogenates after precipitation with 2% perchloric, and using yeast-glutathione reductase, 5, 5' dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH and reading at 340 nm. GSSG was determined by the same method in the presence of 2-vinylpyridine. GSH was calculated from the difference between total glutathione and GSSG[23].

Western-blot analysis of HO-1 expression

Samples of homogenate obtained for HO-1 activity assays were also analyzed by Western immunoblot technique as previously described[24]. An amount of protein (50 μg) from homogenates of control and treated rats was run in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using a 12% acylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK). Separated proteins were transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then probed with polyclonal goat anti-HO-1 antibody (Santa Cruz, BioTech, California)(1:300 dilution in Tris-buffered saline, pH 7.4) overnight at 4℃. Immune complexes were detected using donkey anti goat secondary antibody (1:1500) (Santa Cruz, BioTech, California), and were visualized using ECL reagent (Amersham, Pharmacia). Intensity of bands was analyzed with Gel-Pro® analyzer 3.1 version, Media Cybernetics.

Microscopy and image analysis

Hepatic tissue was fixed in buffered formalin and stained with routine techniques (HE, Reticulin and Masson's trichromic) for light microscopy. For high resolution optic microscopy (HROM) tissue was fixed in 3% glutaraldehyde buffered with sodium cacodylate, embedded in epoxy and stained with toluidine blue. Images from light microscopy were captured and digitised (Cap view) and standardised (PhotoShop 7.0); then the selected sinusoidal areas of the pericentral vein areas were extracted with auto level function. Threshold in red images were obtained through Scion Image B4.02 analyser. Selected areas for quantification were measured as pixels per area (square inches). Standard referenced area utilized was 8.33 square inches[25].

Table 1 Effect of different treatments on portal pressure (mean ± SE)

Treatment	Portal pressure (PP) (mmHg)
Sham	7.9 ± 0.6[b]
PPVL	14.4 ± 1.9
Sham + bilirubin	7.6 ± 0.9[b]
Sham + Sn-PPIX +	7.3 ± 0.7[b]
PPVL + Bilirubin	14.0 ± 1.8
PPVL + Sn-PPIX	13.8 ± 1.6

[b]$P < 0.001$ between sham groups and PPVL, PPVL + Bilirubin and PPVL + Sn-PPIX groups, according to Neuman-Keuls' test.

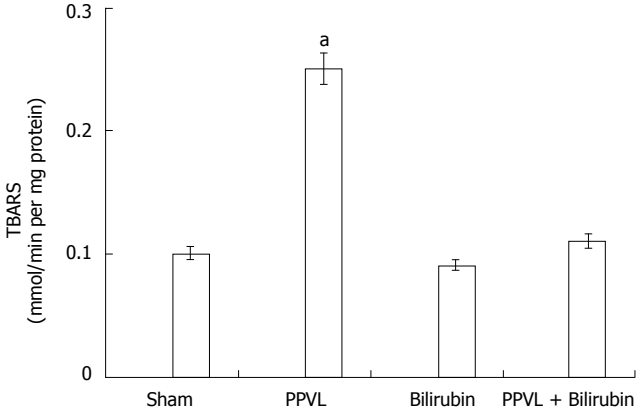

Figure 1 Effect of PPVL and bilirubin on lipid peroxidation. Rats were treated as described in Methods. Values are the means of three different experiments using three rats each time, and bars indicate SE. [a]$P < 0.05$ *vs* sham.

Protein determination

Protein concentration was measured following Lowry *et al*[26] using bovine serum albumin as standard.

Statistical analysis

Results are expressed as mean ± SE. Data were analysed statistically by factorial analysis of variance (ANOVA) and followed by the Neuman-Keuls' test for comparison of means. Differences were considered significant at $P < 0.05$.

RESULTS

Portal pressure measurement

The portal pressure results are shown in Table 1. Differences between Sham *vs* PPVL rats at d 14 without and with bilirubin and Sn-PPIX were significant ($P < 0.001$). No differences were found either in Sham or in PPVL animals when they were treated with bilirubin and Sn-PPIX.

Oxidative stress generation

Reactive oxygen species are responsible for peroxidative cell damage. TBARS were 150% increased in PPVL rat livers (Figure 1).

GSH is a leading substrate for enzymatic antioxidant functions and is also a known radical scavenger. Therefore, if PPVL treatment induced the formation of ROS, it could be expected that GSH levels be affected. It can be

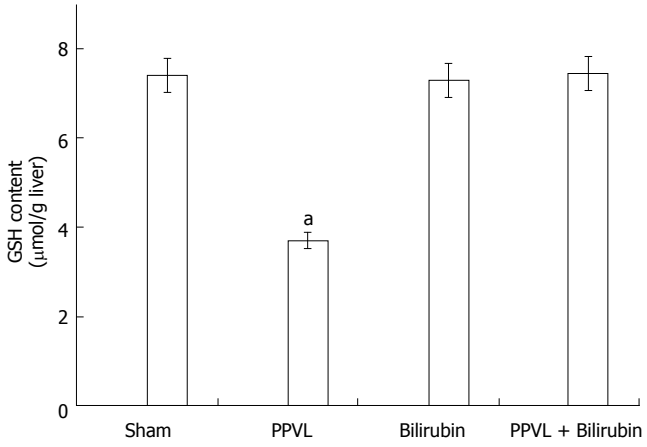

Figure 2 Effect of PPVL and bilirubin on GSH liver content. Rats were treated as described in Methods. Values are the means of three different experiments using three rats each time, and bars indicate SE. $^{a}P < 0.05$ *vs* sham.

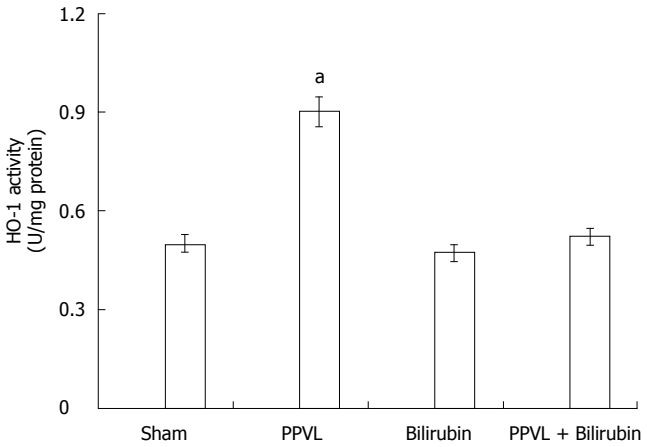

Figure 3 Effect of PPVL and bilirubin on HO-1 activity. Rats were treated as described in Methods. One unit of HO-1 is defined as the amount of enzyme producing 1 nmol of bilirubin per 30 min under the standard incubation conditions. Values are the means of three different experiments using three rats each time, and bars indicate SE. $^{a}P < 0.05$ *vs* sham.

Table 2 Effect of PPVL and bilirubin on antioxidant enzyme activities (mean ± SE)

Group	Catalase (pmol/mg protein)	Total superoxide dismutase (U/mg protein)[1]	Glutathione peroxidase (U/mg protein)[2]
Sham	10.5 ± 1.1[a]	9.0 ± 0.2[b]	0.129 ± 0.004[a]
PPVL	6.5 ± 0.5	4.5 ± 0.1	0.100 ± 0.004
Sham + Bilirubin	10.7 ± 0.9[a]	9.3 ± 0.1[b]	0.132 ± 0.006[a]
PPVL + Bilirubin	9.4 ± 0.9[a]	8.9 ± 0.2[b]	0.131 ± 0.004[a]

Enzymatic activities were assayed as described in the text. [1]One unit of SOD activity is defined as the amount of enzyme required to inhibit 50% of the epinephrine autooxidation. [2]One unit of the enzyme represents the decrease of 1 mmol of NADPH/min under the assay conditions. $^{a}P < 0.05$ between Catalase and Glutathione Peroxidase of Sham groups, PPVL + Bilirubin and PPVL group, $^{b}P < 0.01$ between Superoxide Dismutase of group of Sham groups, PPVL + Bilirubin and PPVL group and PPVL group according to Neuman-Keuls' test.

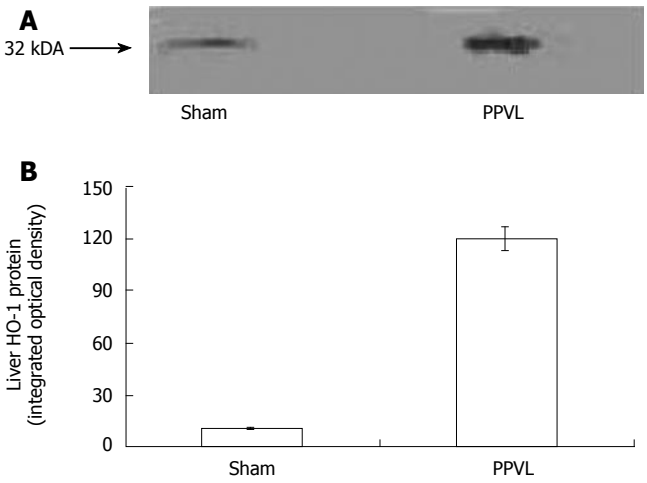

Figure 4 Western blot analysis of HO-1 expression in PPVL rat liver (**A**). Densitometry was done to quantify HO-1 protein expression (**B**). The blot is representative of 3 blots with a total of 4-5 samples/group between the 3 blots. $P < 0.01$ sham *vs* PPVL group.

seen in Figure 2 that in the liver of PPVL animals GSH concentration was about 50% decreased when compared to Sham group. When rats were pretreated with bilirubin both TBARS and GSH levels were equal to Sham animals (Figures 1 and 2).

Enzymatic defence system

Table 2 shows the activities of CAT, SOD and GSH-Px in the liver of Sham and PPVL animals pretreated with bilirubin as well as in both groups without bilirubin. These enzymes were significantly inhibited (CAT 38%, SOD 50% and GSH-Px 23%) in PPVL rats. Pretreatment with bilirubin had no effect in Sham animals, but in PPVL rats the inhibition of antioxidant enzymes was totally prevented.

Heme oxygenase activity and expression

As it is shown in Figure 3, PPVL treatment increased liver HO-1 activity by 80%. Bilirubin pretreatment did not have any effect in Sham rats, but it completely prevented HO-1 induction provoked by PPVL. As occurred with HO-1 activity, a similar increment in HO-1 expression was obtained in liver of PPVL rats (Figure 4A and B).

Previous results using acute intoxication with different drugs demonstrated that as a consequence of ROS production, liver GSH levels rapidly decreased and then HO-1 was induced. It was also observed that HO-1 induction appeared to occur once the ROS was increased and the antioxidant defence system decreased. Bilirubin action as a protective agent against oxidative stress suggests that inhibition of HO-1 could enhance even more the oxidative stress induced by PPVL model. Therefore, the effect of Sn-PPIX, a strong inhibitor of HO-1 was assayed 24 h before the end of the experiment. Table 3 shows that a single administration of Sn-PPIX decreased to half HO-1 activity in Sham rats. PPVL animals pretreated with the inhibitor of HO-1 (Sn-PPIX), slightly decreased hepatic GSH levels (20%) and significantly increased TBARS production (70%) over the values obtained by PPVL treatment.

Effect of CO on hepatic sinusoidal vasodilatation

As it is shown in Table 4 and Figure 5, CO produced a significant increase in the sinusoidal hepatic areas of PPVL rats when compared to sham animals. Pretreatment of

Table 3 Effect of Sn-PPIX administration on the HO-1 induction and on TBARS and GSH contents in PPVL rat livers (mean ± SE)

Treatment	HO-1 (U/mg protein)[1]	TBARS (nmol/min per mg protein)	GSH content (μmol/g liver)
Sham	0.50 ± 0.01[b]	0.10 ± 0.01[d]	7.5 ± 0.2[d]
PPVL	0.90 ± 0.02	0.25 ± 0.01	3.7 ± 0.1
Sham + Sn-PPIX	0.25 ± 0.01	0.11 ± 0.01[d]	7.2 ± 0.3[d]
PPVL + Sn-PPIX	0.48 ± 0.04	0.32 ± 0.02	2.3 ± 0.2

Sn-PPIX was administered as described in Materials and Methods. Sham animals were injected with saline solution. Enzymatic activity was assayed as described in the text. [1]One unit of the enzyme forms 1 nmol of bilirubin/30 min under assay conditions. Different letters within columns indicate significant differences according to Neuman-Keuls' test. Differences in HO-1 were of [b]$P < 0.01$ between Sham group and PPVL, Sham + Sn-PPIX groups. Differences in TBARS and in GSH content were of [d]$P < 0.01$ between Sham groups and PPVL groups.

Table 4 Effect of different treatments on vasodilatation of hepatic sinusoids (mean ± SE)

Treatment	Sinusoidal area
Sham (n = 258)	3.64 ± 0.08
PPVL (n = 245)	4.20 ± 0.02[b]
Sham + Sn-PPIX (n = 257)	3.45 ± 0.01
PPVL + Sn-PPIX (n = 249)	3.48 ± 0.04

The selected area for quantification was measured as pixels per area (square inch). The area standard used was 8.33 square inch. [b]$P < 0.01$ between PPVL and the other three groups, according to Neuman-Keuls' test.

PPVL animals with Sn-PPIX totally prevented this effect and similar values to Sham group were obtained (Table 4). Groups Sham and PPVL + Sn-PPIX showed normal histological features when focusing on sinusoidal space (similar Figure 5A), while group PPVL showed sinusoidal dilation (Figure 5B).

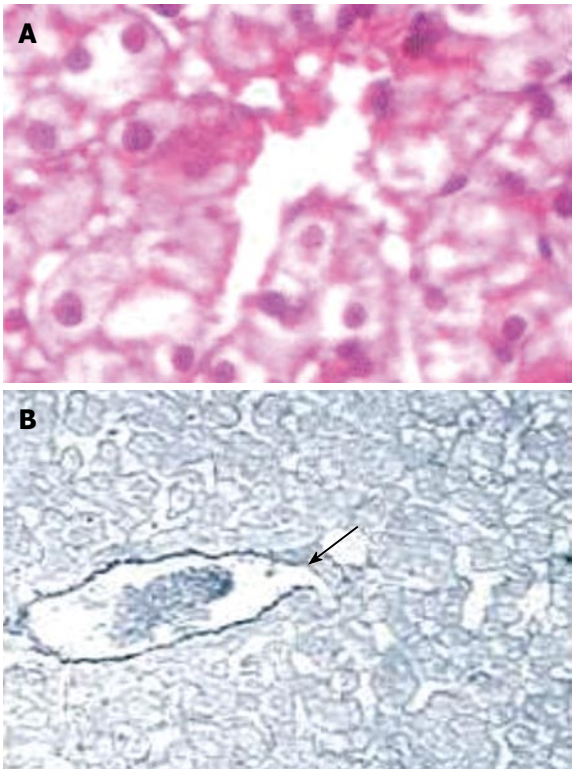

Figure 5 Hepatic centrolobular zone of Sham operated rats (**A**). Sinusoidal area spread normal histological features (HE, 100 x magnification). Hepatic centrolobular zone corresponding to PPVL group (**B**). Sinusoidal area is increased (arrows) (Reticulin, enhanced image, 40 x magnification).

DISCUSSION

Aerobic organisms are continuously exposed to oxygen, which renders them prone to damage generated by ROS. There are a number of cellular mechanisms to protect the cell from oxidative stress. Antioxidant enzymes such as CAT, SOD and GSH-Px play critical roles in oxidative stress protection by converting ROS into less harmful products[27]. Recently, HO-1 and its product bilirubin have gained attention in cytoprotection against oxidant mediated injury[10-14,28,29].

Our results showed that the administration of bilirubin in rats decreased PPVL-induced lipid peroxidation, restored GSH content and activity of the antioxidant enzymatic system to normal levels. These results open possibilities for considering the use of bilirubin as an efficient antioxidant and free radical scavenger (Figures 1 and 2, Table 2).

Bilirubin administration totally prevented the decrease of antioxidant enzyme activities and induction of HO-1 activity provoked by PPVL (Table 2, Figure 3). These findings are in agreement with our own previous reports on the protective effect of bilirubin against oxidative stress caused by different physical and chemical agents[28].

Administration of Sn-PPIX, a known competitive inhibitor of HO-1 expectedly decreased HO-1 activity (Table 3). When the inhibitor was given to PPVL rats, HO-1 activity remained at control levels, but an increase in TBARS content and a decrease in GSH levels were observed (Table 3).

CO is, like nitric oxide, an endogenous compound that activates guanylate cyclase[30], leading to the generation of cyclic guanosine monophosphate, which in turn mediates various physiological functions, and excessive production of CO, a consequence of HO-1 overexpression, could play an important role in modulating vascular tone under different pathological situations[16,31]. Besides, it has been demonstrated that administration of zinc protoporphyrin IX, a strong inhibitor of heme oxygenase, elicits a marked increase in the vascular resistance as a consequence of sinusoidal constriction[32]. These results are in agreement with the effects observed in PPVL rats pretreated with Sn-PPIX (Table 4, Figure 5). Increased production of CO, as a consequence of HO-1 induction, may be beneficial in some pathological situations, including hypertension[33], but can also be detrimental in other disease conditions[34]. For instance, in PH it would be detrimental because an increased production of CO in splanchnic organs may contribute to the development and maintenance of the splanchnic hyperdynamic circulation associated with the PH syndrome. But at this point, we can also speculate that an enhanced release of the vasodilator CO may contribute to the modulation of hepatic blood flow.

It has been demonstrated that HO-1 is induced in

splanchnic organs of PH rats [35-37], and it was also reported that patients with portal hypertensive diseases had significantly greater the activity and expression of liver HO-1 than those from normal individuals[38]. HO-1 activity in the liver is not related to systemic vascular resistance; however this organ may show high activity of HO due to that reticuloendothelial cell-rich tissues are involved in the removal of senescent erythrocytes and plasmatic hemoglobin from the circulation.

The precise mechanisms whereby HO-1 expression is induced in PH rats are still unknown, but the results here obtained lead us to believe that this induction was provoked as a consequence of oxidative stress generation. Besides, it is noteworthy that HO-1 is transcriptionally activated by several chemical and physical factors that may be increased during PH[1], including cytokines, endotoxin and shear stress[39]. For these reasons, in the present study we suggest a beneficial role of HO-1 overexpression in a rat model of prehepatic portal hypertension.

In this study, no differences in portal pressure were observed in PPVL rats when compared this group with PPVL + Bilirubin and PPVL + Sn-PPIX groups. This fact could be explained by the persistence of some amount of portal systemic shunting and by the hepatic blood resistance that was not modified despite the pericentral sinusoidal dilation documented here.

In conclusion, our data suggest that ROS generation by portal hypertension situation alter heme degradation. They also evidenced that the cell possesses an inducible pathway against oxidative stress finally leading to increased bilirubin formation, which then exerts its antioxidant protective action. These results shed light on the response of heme degradation to PPVL-induced oxidative stress, and further support the involvement of bilirubin as a physiological protective agent against PPVL-induced oxidative cell injury. The potential use of bilirubin as an antioxidant in combination with other antioxidants or with conventional treatments, as a new therapeutic approach for portal hypertensive patients, can be considered.

REFERENCES

1 **Bosch J**, Pizcueta P, Feu F, Fernández M, García-Pagán JC. Pathophysiology of portal hypertension. *Gastroenterol Clin North Am* 1992; **21**: 1-14

2 **Evelson P**, Llesuy S, Filinger E, Rodriguez RR, Lemberg A, Scorticati C, Susemihl M, Villareal I, Polo JM, Peredo H, Perazzo JC. Decreased oxidative stress in prehepatic portal hypertensive rat livers following the induction of diabetes. *Clin Exp Pharmacol Physiol* 2004; **31**: 169-173

3 **Di Mascio P**, Murphy ME, Sies H. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am J Clin Nutr* 1991; **53**: 194S-200S

4 **Tenhunen R**, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* 1968; **61**: 748-755

5 **Kutty RK**, Maines MD. Purification and characterization of biliverdin reductase from rat liver. *J Biol Chem* 1981; **256**: 3956-3962

6 **Maines MD**, Kappas A. Studies on the mechanism of induction of haem oxygenase by cobalt and other metal ions. *Biochem J* 1976; **154**: 125-131

7 **Keyse SM**, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 1989; **86**: 99-103

8 **Maines MD**. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* 1988; **2**: 2557-2568

9 **Tomaro ML**, Frydman J, Frydman RB. Heme oxygenase induction by CoCl2, Co-protoporphyrin IX, phenylhydrazine, and diamide: evidence for oxidative stress involvement. *Arch Biochem Biophys* 1991; **286**: 610-617

10 **Llesuy SF**, Tomaro ML. Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochim Biophys Acta* 1994; **1223**: 9-14

11 **Ossola JO**, Tomaro ML. Heme oxygenase induction by UVA radiation. A response to oxidative stress in rat liver. *Int J Biochem Cell Biol* 1998; **30**: 285-292

12 **Clark JE**, Foresti R, Green CJ, Motterlini R. Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress. *Biochem J* 2000; **348** Pt 3: 615-619

13 **Stocker R**. Induction of haem oxygenase as a defence against oxidative stress. *Free Radic Res Commun* 1990; **9**: 101-112

14 **Yamaguchi T**, Terakado M, Horio F, Aoki K, Tanaka M, Nakajima H. Role of bilirubin as an antioxidant in an ischemia-reperfusion of rat liver and induction of heme oxygenase. *Biochem Biophys Res Commun* 1996; **223**: 129-135

15 **Ewing JF**, Maines MD. Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain. *J Neurochem* 1993; **60**: 1512-1519

16 **Otterbein LE**, Choi AM. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**: L1029-1037

17 **Maines MD**, Trakshel GM, Kutty RK. Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J Biol Chem* 1986; **261**: 411-419

18 **Chojkier M**, Groszmann RJ. Measurement of portal-systemic shunting in the rat by using gamma-labeled microspheres. *Am J Physiol* 1981; **240**: G371-G375

19 **Chance B**, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 1979; **59**: 527-605

20 **Flohé L**, Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol* 1984; **105**: 114-121

21 **Misra HP**, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972; **247**: 3170-3175

22 **Buege JA**, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; **52**: 302-310

23 **Anderson ME**. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 1985; **113**: 548-555

24 **Foresti R**, Clark JE, Green CJ, Motterlini R. Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J Biol Chem* 1997; **272**: 18411-18417

25 **Masseroli M**, Caballero T, O'Valle F, Del Moral RM, Pérez-Milena A, Del Moral RG. Automatic quantification of liver fibrosis: design and validation of a new image analysis method: comparison with semi-quantitative indexes of fibrosis. *J Hepatol* 2000; **32**: 453-464

26 **LOWRY OH**, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275

27 **Halliwell B**, Gutteridge JMC. Free Radicals in Biology and Medicine. 2nd Ed. Oxford: Clarendon Press, 1989: 105-161

28 **Tomaro ML**, Batlle AM. Bilirubin: its role in cytoprotection against oxidative stress. *Int J Biochem Cell Biol* 2002; **34**: 216-220

29 **Noriega GO**, Tomaro ML, del Batlle AM. Bilirubin is highly effective in preventing in vivo delta-aminolevulinic acid-induced oxidative cell damage. *Biochim Biophys Acta* 2003; **1638**: 173-178

30 **Wang R**, Wang Z, Wu L. Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *Br J Pharmacol* 1997; **121**: 927-934

31 **Otterbein LE**, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003; **24**: 449-455
32 **Suematsu M**, Goda N, Sano T, Kashiwagi S, Egawa T, Shinoda Y, Ishimura Y. Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J Clin Invest* 1995; **96**: 2431-2437
33 **Motterlini R**, Gonzales A, Foresti R, Clark JE, Green CJ, Winslow RM. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. *Circ Res* 1998; **83**: 568-577
34 **Lores-Arnaiz S**, Perazzo JC, Prestifilippo JP, Lago N, D'Amico G, Czerniczyniec A, Bustamante J, Boveris A, Lemberg A. Hippocampal mitochondrial dysfunction with decreased mtNOS activity in prehepatic portal hypertensive rats. *Neurochem Int* 2005; **47**: 362-368
35 **Fernandez M**, Bonkovsky HL. Vascular endothelial growth factor increases heme oxygenase-1 protein expression in the chick embryo chorioallantoic membrane. *Br J Pharmacol* 2003; **139**: 634-640
36 **Erario MA**, Gonzales S, Romay S, Eizayaga FX, Castro JL, Lemberg A, Tomaro ML. Role of heme oxygenase/carbon monoxide pathway on the vascular response to noradrenaline in portal hypertensive rats. *Clin Exp Pharmacol Physiol* 2005; **32**: 196-201
37 **Eizayaga F**, Scorticati C, Prestifilippo JP, Romay S, Fernandez MA, Castro JL, Lemberg A, Perazzo JC. Altered blood-brain barrier permeability in rats with prehepatic portal hypertension turns to normal when portal pressure is lowered. *World J Gastroenterol* 2006; **12**: 1367-1372
38 **Makino N**, Suematsu M, Sugiura Y, Morikawa H, Shiomi S, Goda N, Sano T, Nimura Y, Sugimachi K, Ishimura Y. Altered expression of heme oxygenase-1 in the livers of patients with portal hypertensive diseases. *Hepatology* 2001; **33**: 32-42
39 **Zuckerbraun BS**, Billiar TR. Heme oxygenase-1: a cellular Hercules. *Hepatology* 2003; **37**: 742-744

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PO Box 2345, Beijing 100023, China
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BASIC RESEARCH

Changes of inducible protein-10 and regulated upon activation, normal T cell expressed and secreted protein in acute rejection of pancreas transplantation in rats

Jun Zhu, Ze-Kuan Xu, Yi Miao, Xun-Liang Liu, Hong Zhang

Jun Zhu, Ze-Kuan Xu, Yi Miao, Xun-Liang Liu, Department of General Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Jun Zhu, Hong Zhang, Department of Experimental Surgery, Xuzhou Medical College, Xuzhou 221004, Jiangsu Province, China
Supported by a grant from the "135" Foundation of Jiangsu Province, No. 2003-19
Correspondence to: Dr. Ze-Kuan Xu, Department of General Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. xuzekuan@hotmail.com
Telephone: +86-25-83718836
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Abstract

AIM: To investigate the role of IFN-γ inducible protein -10 (IP-10) and regulated upon activation, normal T cell expressed and secreted (RANTES) protein in acute pancreatic allograft rejection in rats.

METHODS: An experimental pancreas transplantation model was established using diabetic SD rats as the recipient, induced by applying streptozocin (STZ). Pancreas transplantation was performed with a physiologic method of portal venous and enteric drainage. Rats were divided into two groups, isograft group (group A, *n* = 24) and allograft group (group B, *n* = 24) in which either healthy SD rats or Wistar rats served as donors, respectively. Twelve diabetic or healthy SD rats were used as controls. At d 1, 4, 7, and 10 post transplantation, serum IP-10 and RANTES were assessed by ELISA and their expression in the allografts was determined by immunohistochemistry.

RESULTS: In group B (allograft group), the development of acute rejection was significantly correlated with increased serum concentration and tissue expression of IP-10 and RANTES, with a peak level at d 7 post transplantation. In contrast, there was no obvious change before and after transplantation in group A (isograft group).

CONCLUSION: Our study suggests a possible role of IP-10 and RANTES in acute rejection and early monitoring of chemokines may be helpful in predicting the outcome of pancreas transplantation.

Key words: Pancreas transplantation; Chemokine; Rats

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INTRODUCTION

In the past several decades, great success has been made in the transplantation field. However, the acute rejection is still a tremendous obstacle to the development of the transplantation. Acute allograft rejection is a complex process comprising interrelated series of events, such as the recognition of the allograft antigen, the activation and proliferation of the leukocytes, the migration of the leukocytes to the allograft. In the pathogenic process, several kinds of inflammatory molecules are required, such as proinflammatory cytokines, adherence molecules and chemokines. The chemokines are a large family of "chemoattractant cytokines", including IFN-γ inducible protein-10 (IP-10), regulated upon activation, normal T cell expressed and secreted (RANTES), Mig, *etc*. They play a critical role in directing leukocytes to the allograft and in amplifying intragraft inflammation during rejection[1,2]. In the past years it has become evident that individual proinflammatory chemokines (such as IP-10, RANTES) are indispensable in the rejection process of heart transplantation, kidney transplantation, and lung transplantation, compared to other chemokines[3-5]. However the roles of chemokines in pancreas transplantation are still not well known and pancreas transplantation has its own speciality that differs from other organ transplantations. For example, it is well known that pancreatic grafts are very susceptible to allograft rejection because of the strong immunogenicity of pancreas itself[6]. To assess the changes of chemokines IP-10 and RANTES in the acute rejection of pancreas transplantation, we established the pancreas transplantation model in rats firstly, using a physiologic method of portal

venous and enteric drainage[7], and then evaluated the severity of rejection, detected the concentration of serum RANTES and IP-10 using ELISA kits, and examined the expressive position and intensity of IP-10 and RANTES in the allograft pancreas.

MATERIALS AND METHODS

Animal preparation

The diabetic rats were induced firstly by single intravenous injection of streptozocin (STZ; Sigma, USA) at a dose of 50 mg/kg of body weight, when the closed flock male SD rats weighing 250-280 g were chosen (offered by the Experimental Animal Center of Jiangsu Province, China). After the STZ injection, the nonfasting blood glucose and urine glucose of the rats were assayed before the inducement and measured on alternate day. Only rats with nonfasting blood glucose exceeding 16.8 mmol/L and the strong positive reaction of the urine glucose were selected as recipients. The closed flock male Wistar rats (offered by the Silaike Co. Ltd, Shanghai, China) and healthy SD rats weighing 220-250 g served as donors. The donors and recipients were matched under the condition that the weights of the recipients were more than that of donors by about 30 g.

Grouping of experimental rats

Rats were divided into two groups, isograft group (group A, $n = 24$) and allograft group (group B, $n = 24$), in which either healthy SD rats or Wistar rats served as donors, respectively. Twelve diabetic or healthy SD rats were used as controls.

Surgical procedure and collection of specimen

A physiologic method for pancreas transplantation was adopted, in which the vein was reconstructed by end-to-side anastomosis between the donor portal vein and the recipient superior mesenteric vein, and arterial reconstruction was carried out by end-to-side anastomosis of the donor to the recipient abdominal aorta, and enteric drainage was performed by a side-to-side anastomosis between the duodenum of donors and that of recipients. The level of the recipient's blood glucose below 11.2 mmol/L at 1 d post operation was regarded as successful transplantation. The recipients were sacrificed at 1, 4, 7, 10 d ($n = 6$ animals/time point) after transplantation. The 12 rats in control groups were killed at the beginning of the experiment. Blood samples were collected and placed quietly for clotting for 2 h at room temperature before centrifuging for 30 min at 1000 × g, then the serum was pipetted immediately and stored at -70℃. After representative portions of pancreas grafts were removed, some of them were immediately snap-frozen in liquid nitrogen for immunohistology and the rest were fixed in 10% formalin for histopathological examination.

Histopathology examination

The samples of pancreas grafts were fixed, dehydrated, embedded, sliced, and stained with hematoxylin and eosin following the routine proposal. The classification of acute rejection was stated according to the Nakhleh Classification Criterion[8].

Table 1 Classification of acute rejection in allograft group post transplantation

	Grade Ⅰ	Grade Ⅱ	Grade Ⅲ	Grade Ⅳ
1 d	5	1		
4 d		4	2	
7 d			5	1
10 d				6

Nakhleh Classification Criterion, Grade Ⅰ: no rejection present, Grade Ⅱ: mild lymphoplasmacellular infiltration or endothelialitis, Grade Ⅲ: moderate lymphoplasmacellular infiltration or arteritis/vasculitis, Grade Ⅳ: severe lymphoplasmacellular infiltration or fibrinoid necrosis.

Determination of serum IP-10 and RANTES

ELISA kits (TPI INC., USA) were used for the determination of serum IP-10 and RANTES, and the procedure was strictly according to the protocol recommended by the manufacturers. The results were expressed as the quantity per mL serum.

Immunohistology

For immunohistology, 10 μm frozen sections of pancreas were prepared, fixed in acetone for 10 min, dried in the airy place, and incubated with goat polyclonal IP-10 antibodies and rabbit polyclonal RANTES antibodies respectively. Then, the sections were incubated with rabbit anti-goat IgG and goat anti-rabbit IgG respectively. All the reagents were offered by Santa Cruz Co, USA. The cells stained clearly were regarded as positive ones. According to the percentage of positive cells in the whole infiltrating immune cells, the results of immunohistology were expressed in four grades: negative (the rate of positive cells < 5%), mild positive (the rate of positive cells > 5% and < 25%), moderate positive (the rate of positive cells > 25% and < 50%), strong positive (the rate of positive cells > 50%).

Statistical analysis

The concentration of serum IP-10 and RANTES were expressed as mean ± SD. The significance of differences was tested using either *t*-test for means of two samples or analysis of Variance and q-test for means of multiple samples. $P < 0.05$ was considered as significant.

RESULTS

Classification of acute rejection

The acute rejection was classified according to the criterion stated by Nakhleh. In this study, a mild edema appeared around the islet and the acinus 1 d post transplantation both in the allograft and isograft groups. The edema disappeared and no evident rejection was found at 4, 7, and 10 d after the operations in the isograft group, though evident rejection appeared in the allograft group (Table 1).

Changes of serum IP-10 and RANTES

In the allograft group, serum IP-10 concentration was elevated significantly since 4 d and peaked at 7 d after the

Table 2 Concentration of serum IP-10 in isograft, allograft and control groups (ng/L)

	1 d	4 d	7 d	10 d
Isograft group	40.88 ± 10.76	36.74 ± 10.33	38.13 ± 12.73	31.83 ± 7.55
Allograft group	43.34 ± 15.29	66.26 ± 11.08[a]	83.28 ± 16.44[a]	70.08 ± 17.65[a]
Control group	28.76 ± 7.41			

[a]$P < 0.05$ *vs* control group.

Table 3 Concentration of serum RANTES in isograft, allograft and control groups (ng/L)

Group	1 d	4 d	7 d	10 d
Isograft	357.87 ± 63.26[a]	274.77 ± 58.22	265.87 ± 43.40	267.55 ± 48.91
Allograft	388.48 ± 104.45[a]	586.72 ± 90.06[a]	746.28 ± 92.64[a]	631.49 ± 106.34[a]
Control	251.18 ± 44.94			

[a]$P < 0.05$ *vs* control group.

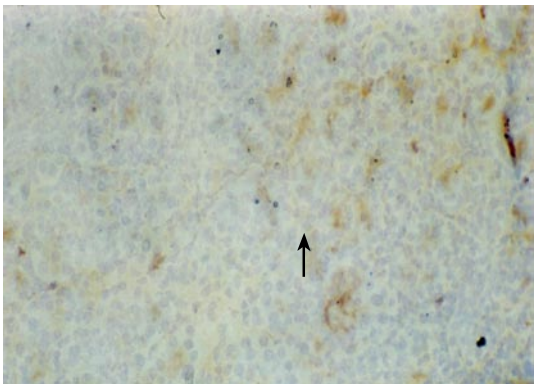

Figure 1 IP-10 immunohistochemical staining of pancreas allografts. Mild expression was observed at 1 d post transplantation (× 200).

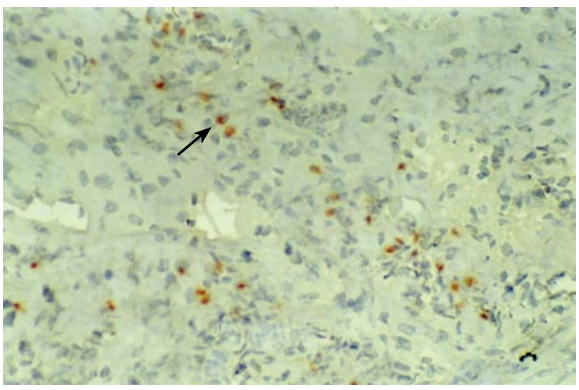

Figure 2 RANTES immunohistochemical staining of pancreas allografts. Mild expression was observed at 1 d post transplantation (× 200).

operations, compared to the control group ($P < 0.05$). However, no significant difference was found between the isograft group and the control group at the four corresponding phases. The tendency of serum RANTES was similar to that of IP-10, only showing a sharp increase at 1 d after the transplantation in the isograft group ($P <$

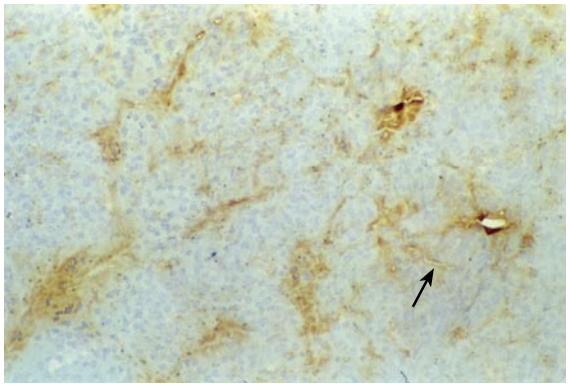

Figure 3 IP-10 immunohistochemical staining of pancreas allografts. Moderate expression was observed at 4 d post transplantation (× 200).

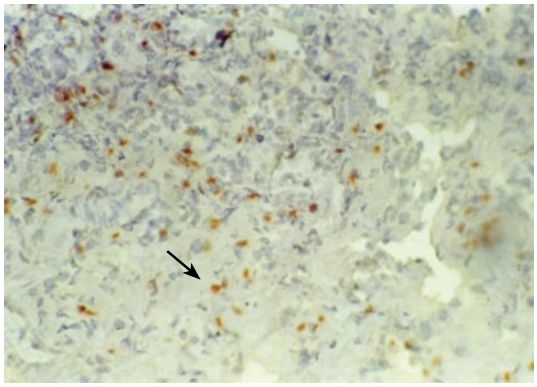

Figure 4 RANTES immunohistochemical staining of pancreas allografts. Moderate expression was observed at 4 d post transplantation (× 200).

0.05) compared with the control group (Tables 2 and 3).

Expression of IP-10 and RANTES in the pancreas grafts

There were no detectable expressions of IP-10 and RANTES protein in the normal pancreas. Mild expression was observed at 1 d after the operation both in the allografts and isografts (Figures 1 and 2). At 4 d after the transplantation, expression of IP-10 and RANTES appeared moderately in the allografts (Figures 3 and 4), whereas their expression could not be detected in the isografts. At 7 d after the operation, the positive expression was strongest in the allografts (Figures 5 and 6). Besides, they were expressed in different places. IP-10 was gathered around the vessels; RANTES was clearly expressed in the lymphocytes in interstitial tissues and concentrated in the narrow space between the inflammatory and normal tissues.

DISCUSSION

Acute rejection is a very complicated process, in which the cellular and humoral immune mechanisms are involved. In the process of acute rejection, the cellular immune reaction is of primary importance[9]. The circulating lymphocytes firstly adhere to the vessel endothelium, then penetrate the vessel wall, cross the interstitial tissue, migrate into the graft, and finally destroy the graft[10]. In these processes, the chemokines are indispensable.

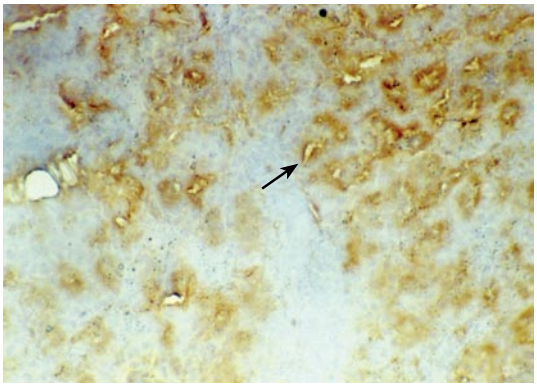

Figure 5 IP-10 immunohistochemical staining of pancreas allografts. Strong positive expression was observed at 7 d post transplantation (× 200).

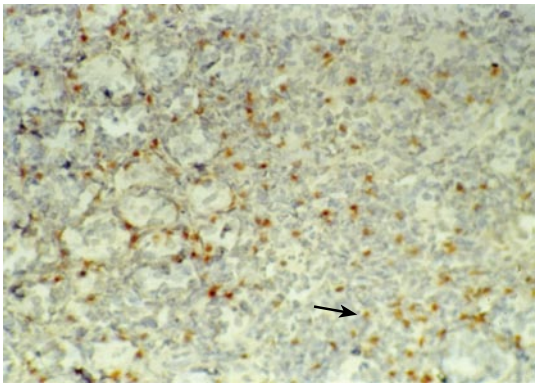

Figure 6 RANTES immunohistochemical staining of pancreas allografts. Strong positive expression was observed at 7 d post transplantation (× 200).

Chemokines accelerate the migration and activation of the leucocytes and lymphocytes through the interaction between the chemokines and their receptors, and play a key role in recruitment of inflammatory cells into an organ transplant[11].

Chemokines are a group of 8-11 kDa proteins, owning four conservative tyrosines in their primary sequence. According to the positional relation between the first tyrosine and the second one, the family are divided into four subfamilies, namely, CXC subfamily, CC subfamily, C subfamily and CX_3C subfamily[12].

IP-10 is one of CXC subfamily, mainly coming from activated fibroblasts, monocytes, endothelial cells and T cells[13]. By the interaction between IP-10 and its receptor (CXCR3), IP-10 function in the recruitment of activated Th1 cells specifically and amplify the Th1 reaction. It has been reported that IP-10 and CXCR3 were detected in the allograft in the lung and heart transplantation, and the quantities of IP-10 and CXCR3 are positively related with the severity of rejection response[14]. The latest study performed by Kanmaz *et al*[15] showed an important correlation between urinary excretion of IP-10 and acute rejection in baboon kidney transplantation and indicated that urinary IP-10 might be a more accurate predictive parameter than serum creatinine to monitor the occurrence of acute rejection of renal transplant.

RANTES belongs to the CC subfamily, produced and secreted by the CD8+ T cells, endothelial cells, and fibroblasts, directing the recruitment of T cells, monocytes, eosinophils to the local tissue[16]. It has been confirmed that RANTES is closely related to the pathogenesis of rejection in heart, lung, kidney transplantation, *etc*. Mulligan *et al*[2] found that the protein of RANTES was detectable 6-8 d after the heart transplantation in mice in heterogeneous group. However, the results were opposite in the homogeneous heart transplantation. Sekine *et al*[17] reported that the mRNA of RANTES was closely positively correlated with the number of infiltrating monocytes, using the models of lung transplantation. Similar results were also obtained in liver transplantation[18]. Some experiments have succeeded in prolonging the functional time of the allografts by inhibiting the interaction between the chemokines and their receptors. Hancock *et al*[19] reported that the survival phase was elongated, provided that the IP-10 knockout mice served as the donors. Yun *et al*[20] demonstrated that the use of Met-RANTES, the specific antagonist to RANTES, could alleviate the infiltration of CD4+ and CD8+ lymphocytes and attenuate allograft rejection in mice. It seems that IP-10 and RANTES play a pivotal role in the rejection of organ transplantation.

In our experiments, there was no detection of IP-10 and RANTES protein in the normal pancreas. In the isograft group, the proteins of IP-10 and RANTES were detected weakly at 1 d, and restored to the normal level since 4 d post transplantation. In the allograft group, the expression of IP-10 and RANTES protein were also upregulated in the primary phase of acute rejection, and then developed with the severity of the rejection reaction. The trend of serum IP-10 and RANTES was the same as that in the graft. It seems that the detection of IP-10 and RANTES benefits the early diagnosis of acute rejection.

We also noticed that the expressing loci and pattern of IP-10 and RANTES was obviously different. IP-10 was gathered around the vessels; meanwhile, RANTES was clearly expressed in the lymphocytes in the interstitial tissues and crowded in the narrow space between the inflammatory and normal tissues. This result perhaps demonstrated that IP-10 and RANTES play different roles in the acute rejection. IP-10 is a key molecule in directing the lymphocytes to penetrate the endothelium and migrate across the tissue. RANTES, produced by the cells in the inflammatory tissues, can be recognized and bound with its receptors on the surface of passage macrophage, T cells. The activated T cells unregulated the expression of RANTES, and more inflammatory cells were attracted into the graft. The process was cycled and amplified and the inflammation aggravated.

In summary, IP-10 and RANTES play a critical role in the acute rejection after pancreas transplantation. Further research will help the understanding of the pathogenesis of acute rejection. IP-10 and RANTES will be a tool for the early diagnosis and a target for the therapy of acute rejection.

REFERENCES

1 **Melter M**, Exeni A, Reinders ME, Fang JC, McMahon G, Ganz P, Hancock WW, Briscoe DM. Expression of the chemokine

receptor CXCR3 and its ligand IP-10 during human cardiac allograft rejection. *Circulation* 2001; **104**: 2558-2564
2 **Mulligan MS**, McDuffie JE, Shanley TP, Guo RF, Vidya Sarma J, Warner RL, Ward PA. Role of RANTES in experimental cardiac allograft rejection. *Exp Mol Pathol* 2000; **69**: 167-174
3 **Fahmy NM**, Yamani MH, Starling RC, Ratliff NB, Young JB, McCarthy PM, Feng J, Novick AC, Fairchild RL. Chemokine and chemokine receptor gene expression indicates acute rejection of human cardiac transplants. *Transplantation* 2003; **75**: 72-78
4 **Segerer S**, Alpers CE. Chemokines and chemokine receptors in renal pathology. *Curr Opin Nephrol Hypertens* 2003; **12**: 243-249
5 **Belperio JA**, Strieter RM. Chemokines/chemokine receptors play an important role in the continuum of acute to chronic lung allograft rejection. *Curr opinn organ transplant* 2004; **9**: 350-360
6 **Ito T**, Uchikoshi F, Tori M, Miao G, Tanaka S, Maeda A, Akamaru Y, Matsuda H, Nozawa M. Immunological characteristics of pancreas transplantation: review and our experimental experience. *Pancreas* 2003; **27**: 31-37
7 **Zhu J**, Xu ZK, Miao Y. Improved method for pancreaticoduodenal transplantation model in rats. *Nanjing Yike Daxue Zazhi* 2004; **18**: 308-311
8 **Nakhleh RE**, Sutherland DE, Tzardis P, Schechner R, Gruessner RW. Correlation of rejection of the duodenum with rejection of the pancreas in a pig model of pancreaticoduodenal transplantation. *Transplantation* 1993; **56**: 1353-1356
9 **Zhang AB**. Pathogenesis of the acute allograft rejection. *Guowai Yixue* (Mianyi Fence) 2004; **27**: 4-7
10 **Le Moine A**, Goldman M, Abramowicz D. Multiple pathways to allograft rejection. *Transplantation* 2002; **73**: 1373-1381
11 **Melter M**, Exeni A, Briscoe DM. Chemokines and their receptors in human clinical solid organ transplantation. *Curr Opin Organ Transplant* 2002; **7**: 77-84
12 **Nelson PJ**, Krensky AM. Chemokines, chemokine receptors, and allograft rejection. *Immunity* 2001; **14**: 377-386
13 **Inston NG**, Cockwell P. The evolving role of chemokines and their receptors in acute allograft rejection. *Nephrol Dial Transplant* 2002; **17**: 1374-1379
14 **Agostini C**, Calabrese F, Rea F, Facco M, Tosoni A, Loy M, Binotto G, Valente M, Trentin L, Semenzato G. Cxcr3 and its ligand CXCL10 are expressed by inflammatory cells infiltrating lung allografts and mediate chemotaxis of T cells at sites of rejection. *Am J Pathol* 2001; **158**: 1703-1711
15 **Kanmaz T**, Feng P, Torrealba J, Kwun J, Fechner JH, Schultz JM, Dong Y, Kim HT, Dar W, Hamawy MM, Knechtle SJ, Hu H. Surveillance of acute rejection in baboon renal transplantation by elevation of interferon-gamma inducible protein-10 and monokine induced by interferon-gamma in urine. *Transplantation* 2004; **78**: 1002-1007
16 **Yun JJ**, Fischbein MP, Laks H, Irie Y, Espejo ML, Fishbein MC, Berliner JA, Ardehali A. Rantes production during development of cardiac allograft vasculopathy. *Transplantation* 2001; **71**: 1649-1656
17 **Sekine Y**, Yasufuku K, Heidler KM, Cummings OW, Van Rooijen N, Fujisawa T, Brown J, Wilkes DS. Monocyte chemoattractant protein-1 and RANTES are chemotactic for graft infiltrating lymphocytes during acute lung allograft rejection. *Am J Respir Cell Mol Biol* 2000; **23**: 719-726
18 **Schenk M**, Zipfel A, Schulz C, Becker HD, Viebahn R. RANTES in the postoperative course after liver transplantation. *Transpl Int* 2000; **13** Suppl 1: S147-S149
19 **Hancock WW**, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med* 2000; **192**: 1515-1520
20 **Yun JJ**, Whiting D, Fischbein MP, Banerji A, Irie Y, Stein D, Fishbein MC, Proudfoot AE, Laks H, Berliner JA, Ardehali A. Combined blockade of the chemokine receptors CCR1 and CCR5 attenuates chronic rejection. *Circulation* 2004; **109**: 932-937

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PO Box 2345, Beijing 100023, China
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BASIC RESEARCH

Clinicopathological and immunohistochemical analysis of gastrointestinal stromal tumor

Feng-Yu Liu, Ji-Ping Qi, Feng-Lin Xu, Ai-Ping Wu

Feng-Yu Liu, Department of Research, Harbin Medical University, Harbin 150086, Heilongjiang Province, China
Ji-Ping Qi, Feng-Lin Xu, Ai-Ping Wu, Department of Pathology, First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Correspondence to: Ji-Ping Qi, Department of Pathology, First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China. qijiping2003@163.com
Telephone: +86-451-88775468
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Abstract

AIM: To investigate the clinicopathological features of gastrointestinal stromal tumor (GIST) and to study the reference indexes for malignancy.

METHODS: Fifty-two cases of primary GIST were distinguished from a group of gastrointestinal mesenchymal tumors using a panel of antibodies such as CD117 and CD34 by immunohistochemical SP method. Their biological behaviors were analyzed using the expression of p21WAF1 and Bax in 52 cases of GIST.

RESULTS: Grossly, the tumor size was between 1.5 cm and 13 cm (mean: 5.5 cm). Focal areas of hemorrhage, necrosis, or small cyst formation could be seen. Microscopically, the tumor was composed of spindle cells (20 cases), epithelioid cells (20 cases) and mixed cells (12 cases). Immunohistochemically, CD117 and CD34 showed diffuse strong positive expressions, the positive rates were 98.1% and 92.3%. SMA, S-100, NSE, NF and MBP showed focal positive expressions, the positive rates were 48.1%, 28.8%, 25%, 21.2% and 42.3% respectively. Vimentins were all positive desmin and CgA were all negative. In normal adult stomach and intestine, the immunoreactive staining for CD117 and CD34 showed immunoreactive interstitial cells of Cajal in myenteric neuroplexus. Among the 52 cases of GIST, 27 were positive for p21WAF1 (51.9%), 29 for Bax (55.8%). The expression of p21WAF1 and Bax had no significent difference with the localization, size, histological subtype of GIST, but had a significent difference with the histological grade (P = 0.000, respectively). p21WAF1 expression had a positive correlation to Bax expression (r = 0.461, P = 0.001, κ = 0.459).

CONCLUSION: GIST has complicated arrangements and various cell types. Positivity of CD117 and CD34 is the most valuable factor in diagnosing GIST. Expression of p21WAF1 and Bax plays an important role in potential malignancy and malignancy rather than in benign GIST. p21WAF1 and Bax may be used as the markers in the assessment of GIST malignant potential.

Key words: Gastrointestinal stromal tumor; p21WAF1; Bax

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the digestive tract[1]. Mutational activation of *c-kit* has been found to be associated with the pathogenesis of GIST[2], CD117 positivity is the most valuable marker in diagnosing GIST. But the biological behaviors of GIST are difficult to predict[3], some metastasize whereas others remain asymptomatic for years[4]. In this study, we detected CD117, CD34, SMA, S-100, NSE, NF, MBP, vimentin, desmin, CgA, p21WAF1 and Bax using immunohistochemical staining method to explore the expression of p21WAF1 and Bax and its correlation to clinicopathologic characteristics of GIST.

MATERIALS AND METHODS

Specimens

Fifty-two cases of GIST were selected from the Department of Pathology, First Hospital of Harbin Medical University in 2001 to 2004. The slides stained with hematoxylin and eosin were reviewed. Based on the diagnostic criteria proposed by Haber *et al*[5], among the 52 cases of GIST, 20 were cases of benign GIST, 12 and 20 were cases of potentially malignant and malignant GIST respectively. Age ranged 34-78 years (mean: 54.3 years).

Immunohischemistry

Resected specimens were fixed in 40 g/L formaldehyde and embedded in paraffin. Four-μm thick sections were dewaxed, rehydrated in graded alcohols, and processed

Table1 Primary antibody used for immunohistochemical study

Antibody	Clone	Dilution	Pretreatment
CD117	polyclonal	instant	Microwave
CD34	QBEnd/10	instant	Microwave
SMA	$1A_4$	instant	None
Vimentin	V9	instant	None
Desmin	ZC18	instant	None
S-100	4C4.9	instant	None
NSE	E27	instant	None
NF	DA2/FNP7	instant	Microwave
MBP	polyclonal	instant	None
CgA	LK2H10	instant	Microwave
p21WAF1	4D10	1:25	Microwave
Bax	2D2	1:50	Microwave

using immunohistochemical SP method. All antibodies were purchased from Beijing Zhongshan Biotechnology CO. LTD (Table 1). Tissues positive for all the purchased antibodies were used as positive controls, sections prepared with PBS instead of the primary antibody were used as negative controls. When the number of positive cells was < 10%, 10%-50%, or > 50%, the immunoreactivity for p21WAF1 and Bax was scored as 1 +, 2 +, 3 +, respectively. When the number of positive cells was ≤ 50% and > 50%, the immunoreactivity for other antibodies was scored as 1 +, 2 +, and 3 +, respectively.

Statistical analysis

Statistical analyses were performed using SPSS 11.5 software. Pearson χ^2 test, Fisher's exact test, Spearman rank correlation test and Kappa test were used when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Grossing findings

The tumor size was 1.5-13 cm (mean: 5.5 cm). Tumors were generally round or oval in shape with pink-white and firm well-circumscribed and cut surface. Focal areas of hemorrhage, necrosis, or small cyst formation could be seen. Submucosal or subserosal tumors sometimes extended into the gastrointestinal lumen, leading to ulceration in mucosa.

Light microscopic findings

Among the 52 cases of GIST, spindle cell type was found in 20, epithelioid cell type in 20 (Figure 1A) and mixed type in 12. The tumor cells arranged in interlacing fascicles or formed whirls. The tumor cells were spindle, oral or round in shape, sometimes signet-ring like cells could be observed with a clear cytoplasm (Figure 1B). Hemorrhage and/or necrosis and/or hyaline degeneration (Figure 1C) could be found in some cases. Mucosal or serosal invasion (Figure 1D) sometimes could be seen in some malignant GISTs.

Table 2 Relation between p21WAF1, Bax expression and clinicopathological features

Items		*n*	p21WAF1			Bax		
			positive	%	*P*	positive	%	*P*
Localization	Stomach/ Intestine	36	17	47.2	0.309	18	50	0.209
	Others	16	10	62.5		11	68.8	
Tumor size	< 5 cm	21	12	57.1	0.535	13	61.9	0.463
	≥ 5 cm	31	15	48.4		16	51.6	
Histological subtype	Spindle	20	11	55	0.720	9	45	0.086
	Epitheloid	20	9	45		10	50	
	Mixed	12	7	58.3		10	83.3	
Histological grade	Benign	20	1	5	0.000	2	10	0.000
	Potentially malignant	12	9	75[b]		11	91.7[b]	
	Malignant	20	17	85		16	80	

[b]$P < 0.01$.

Table 3 Correlation between expressions of p21WAF1 and Bax

p21WAF1	*n*	Bax		*r*	*P*
		+	-		
+	27	21	6	0.461	0.001
-	25	8	17		

Immunohistochemical findimgs

CD117 and CD34 showed diffuse positive expressions, the positive rates were 98.1% (Figure 2A) and 92.3%. SMA, S-100, NSE, NF and MBP showed focal positive expressions, the positive rates were 48.1%, 28.8%, 25%, 21.2% and 42.3%, respectively. Vimentins were all positive while desmin and CgA were all negative. In normal adult stomach and intestine, the immunoreactive staining for CD117 and CD34 showed immunoreactive interstitial cells of Cajal in myenteric neuroplexus (Figure 2B). Among the 52 cases of GIST, 27 were positive for p21WAF1(51.9%, Figure 2C), 29 for Bax (55.8%, Figure 2D).

According to the χ^2 test, the expression of p21WAF1 and Bax had no significant differences in the localization, size, and histological subtype of GIST, but there was a significant difference in the histological grade ($P = 0.000$, Table 2). There was a significant difference between benign and potentially malignant or malignant GISTs, but the difference in p21WAF1 and Bax expression between potentially malignant and malignant GISTs was not significent. According to Spearman rank correlation test and Kappa test, p21WAF1 expression had a positive correlation to Bax expression ($r = 0.461$, $P = 0.001$, $\kappa = 0.459$, Table 3).

DISCUSSION

In 1983, Mazur and Clark[6] first introduced the vague term 'gastrointestinal stromal tumor'. Under light microscope,

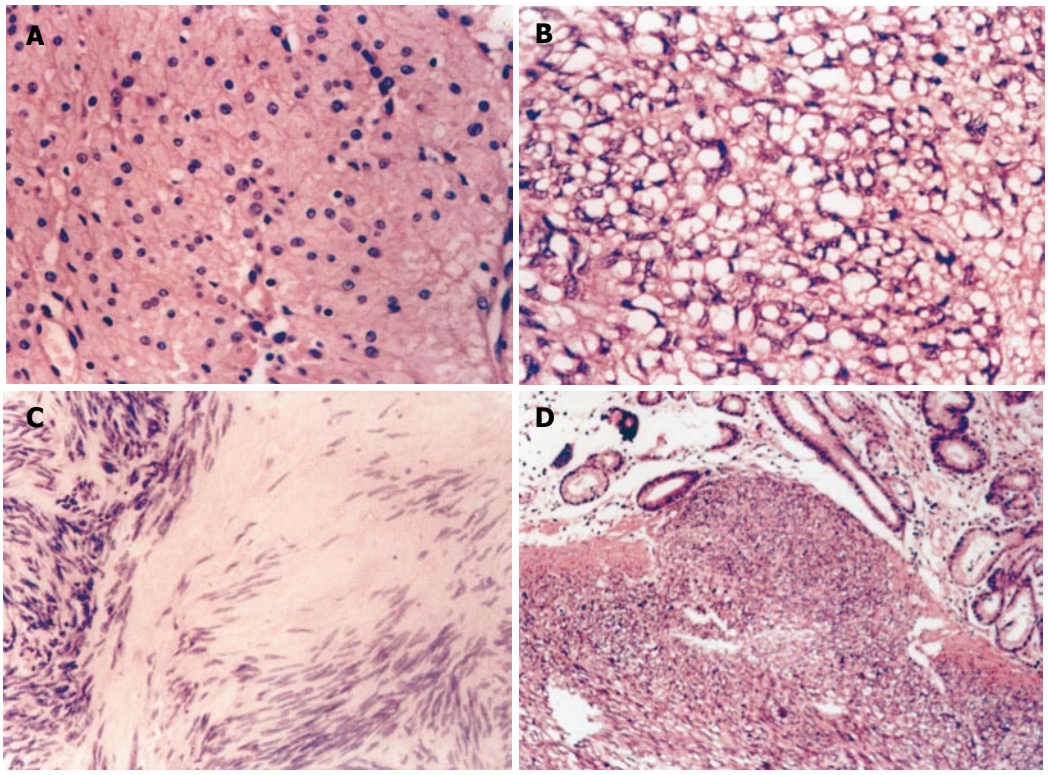

Figure 1 Epithelioid cells (**A**), signet-ring like cells (**B**), hyaline degeneration (**C**), and mucosal invasion (**D**) in GISTs.

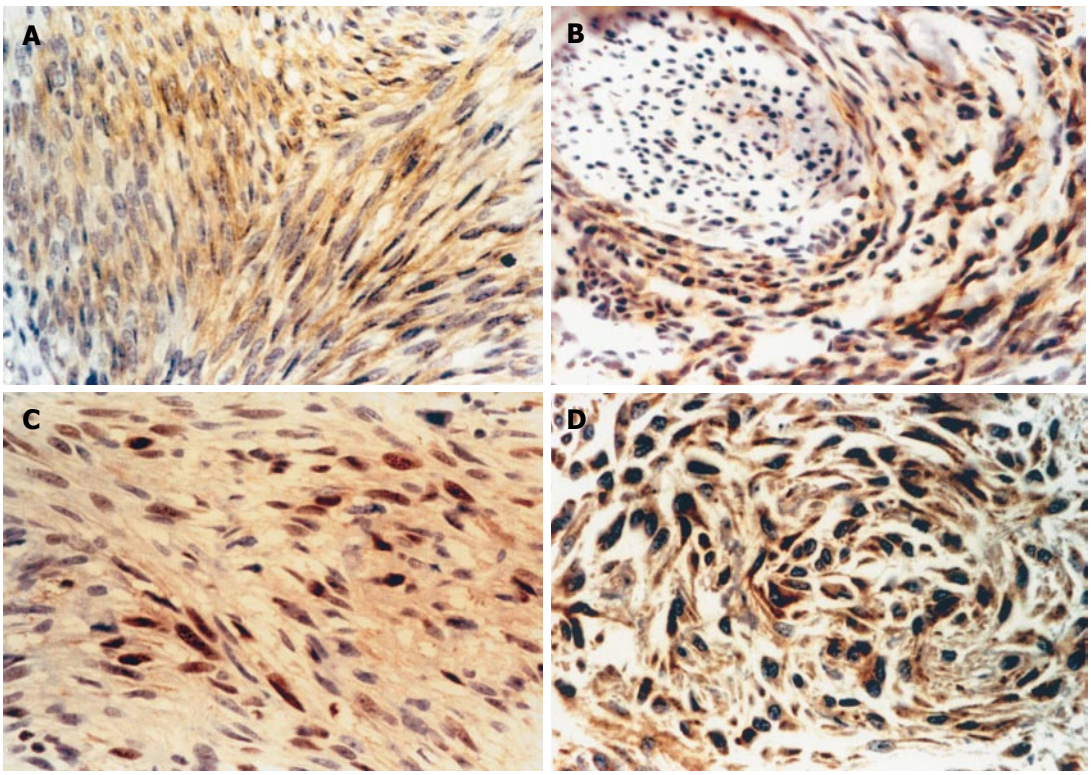

Figure 2 Expressions of CD117 (**A**), CD34 (**B**), p21WAF1 (**C**), and Bax (**D**) in GIST.

the morphology of stromal tumors looks sometimes like a leiomyoma, sometimes like a Schwannoma[7]. Most gastrointestinal mesenchymal tumours, previously classified as leiomyomas, schwannomas or leiomyosarcomas, are today classified as GISTs on the basis of molecular and immunohistological features[8]. GIST derives from the interstital cells of Cajal (ICC), or from a common precursor of ICC and smooth muscle cells of the digestive tract[9]. Immunoperoxidase staining can show both c-kit and CD34-positive cells surrounding the Auerbach ganglia plexus in the gastrointestinal tract[10]. The great majority of GISTs occur in the stomach (60%-70%) and small intestine (25%-35%)[11]. In this study, 46.1% GISTs occurred in stomach and 23.1% in small intestine. In addition, the tumors arose in the esophagus, mesentery. The 52 cases of GISTs were subclassified as spindle or

epithelioid type stromal tumors based on the predominant pattern[12]. Among them, spindle cell type was found in 20, epithelioid cell type in 20 and mixed type in 12.

To make a diagnosis of GISTs, immunohistochemical staining of the CD117 and CD34 is required, because they can characteristically express CD34 and CD117[13]. In this study, CD117 and CD34 showed diffuse positive expressions, the positive rates were 98.1% and 92.3%. Eighteen cases with focal immunoreactivity for SMA were diagnosed as GISTs with smooth muscle differentiation. S-100, NSE, NF and MBP showed focal positive expressions, which could be used as the diagnostic criteria of GISTs with nerve differentiation.

As a sensitive and specific marker of GIST, *c-kit* seems to be a useful antibody in diagnosis and differential diagnosis of GIST, but it may not be used as a prognostic index[14]. Coagulative necrosis, mitotic activity over 10/50HPF, high cellularity with obvious pleomorphism are also helpful parameters for diagnosis of malignancy aside from metastasis and invasion. Adhesion over 5 cm in diameter and mitotic activity over 5/50HPF but less than 10/50HPF might be the potentially malignant parameters[15]. But the effective and reproducible diagnostic parameters for differentiating benign from malignant gastrointestinal stromal tumors (GISTs) are still not clear[16].

p21WAF1 is a cyclin-dependent kinase inhibitor (CDKI) which contributes to the regulation of cell cycle progression by controlling CDK activity and induces a G1 arrest[17,18]. Thus, it is a tumor suppressor gene and likely plays an important role in tumor development. Moreover, reduced expression of p21WAF1 has been reported to have a prognostic value in several human malignancies[19]. Pindzola *et al*[18] reported that malignant gastrointestinal stromal tumor expresses p21WAF1/CIP1. In this study, p21WAF1 expression was not associated with the localization, size and histological subtype of GISTs, except for the tumor grade showing a higher frequency of p21WAF1 expression in potential malignancy and malignancy than that in benign GISTs (75%, 85% and 5%, respectively), indicating that p21WAF1 expression plays an important role in potential malignancy and malignancy rather than in benign GISTs. However, there was no statistical significance between potentially malignant and malignant GISTs, suggesting that overexpression of p21WAF1 is associated with increasing malignant potential, and that p21WAF1 overexpression may be another useful marker in the assessment of the malignant potential in GIST.

The Bcl-2 protein family plays an important role in the regulation of apotosis. This family contains both proapoptotic members (Bax, Bid, Bad, and Bak) and antiapoptotic members (Bcl-2 and Bcl-xl[20]. Overexpression of Bax protein increases apoptosis[21]. Previous studies have shown that Bax expression might be involved in differentiation/histological types of colorectal cancer[22]. Chao *et al*[23] reported that in endometrial carcinoma, the positive rate of Bax overexpression increases correspondingly with increase in histological grade. Although apoptosis is associated with the tumor grade of various carcinomas, little is understood about the association of apoptosis in mesenchymal tumors[24]. Noguchi *et al*[25] reported that there is no statistically significant difference in Bax expression between benign and malignant tumors. In our study, Bax expression was associated with tumor grade showing a higher frequency of Bax expression in potential malignancy and malignancy than in benign GISTs (91.7%, 80%, 10%, respectively), suggesting that Bax expression plays an important role in potential malignancy and malignancy rather than in benign GISTs. However, there was no statistical significance between potential malignancy and malignancy, Suggesting that overexpression of Bax is associated with increasing malignant potential. Thus Bax overexpression may be another useful marker in assessment of the malignant potential in GISTs.

Yang *et al*[26] reported that p21WAF1/CIP1 could inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells, and that inhibition of VSMC growth by overexpression of human p21 gene is accompanied with induction of apoptosis. These results suggest that regulation of cell cycle by p21 may be closely linked to programmed cell death /apoptosis in human vascular smooth muscle cells[27], but a number of recent studies have pointed out that in addition to being an inhibitor of cell proliferation, p21WAF1 acts as an inhibitor of apoptosis[28,29]. In our study, a positive correlation was found between p21WAF1 and Bax ($r = 0.461$, $\kappa = 0.459$), demonstrating that p21WAF1 is closely linked to Bax. In conclusion, p21 gene induces apoptosis by increasing Bax expression and plays an important role in potential malignant and malignant GISTs. Moreover, other factors besides p21WAF1 may regulate Bax.

REFERENCES

1 **Duensing A,** Heinrich MC, Fletcher CD, Fletcher JA. Biology of gastrointestinal stromal tumors: KIT mutations and beyond. *Cancer Invest* 2004; **22**: 106-116

2 **Kim TW,** Lee H, Kang YK, Choe MS, Ryu MH, Chang HM, Kim JS, Yook JH, Kim BS, Lee JS. Prognostic significance of c-kit mutation in localized gastrointestinal stromal tumors. *Clin Cancer Res* 2004; **10**: 3076-3081

3 **Gelen T**, Elpek GO, Aksoy NH, Ogüş M, Keleş N. p27 Labeling index and proliferation in gastrointestinal stromal tumors: correlations with clinicopathologic factors and recurrence. *Jpn J Clin Oncol* 2003; **33**: 346-352

4 **Tornóczky T**, Kövér E, Pajor L. Frequent occurrence of low grade cases among metastatic gastrointestinal stromal tumours. *J Clin Pathol* 2003; **56**: 363-367

5 **Haber MH,** Gattuso P, Spitz DJ, David O. Differential diagnosis in surgical pathology. 1st ed. Beijing: Health Science Asia, Elsevier Science, 2002: 188-192

6 **Mazur MT,** Clark HB. Gastric stromal tumors. Reappraisal of histogenesis. *Am J Surg Pathol* 1983; **7**: 507-519

7 **Liu P**, Na J, Wang Y, He Q, Zhang Y, Tang X, Zou W. Study of gastrointestinal stromal tumors by light microscopy, electron microscopy and immunohistochemistry. *Zhonghua Binglixue Zazhi* 2002; **31**: 199-203

8 **Bucher P**, Villiger P, Egger JF, Buhler LH, Morel P. Management of gastrointestinal stromal tumors: from diagnosis to treatment. *Swiss Med Wkly* 2004; **134**: 145-153

9 **Ray-Coquard I**, Le Cesne A, Michallet V, Boukovinas I, Ranchere D, Thiesse P, Baty V, Blay JY. [Gastro-intestinal stromal tumors: news and comments]. *Bull Cancer* 2003; **90**: 69-76

10 **Wang L,** Vargas H, French SW. Cellular origin of gastrointestinal stromal tumors: a study of 27 cases. *Arch*

Pathol Lab Med 2000; **124**: 1471-1475
11 **Miettinen M**, Majidi M, Lasota J. Pathology and diagnostic criteria of gastrointestinal stromal tumors (GISTs): a review. *Eur J Cancer* 2002; **38** Suppl 5: S39- S51
12 **Ma CK,** Amin MB, Kintanar E, Linden MD, Zarbo RJ. Immunohistologic characterization of gastrointestinal stromal tumors: a study of 82 cases compared with 11 cases of leiomyomas. *Mod Pathol* 1993; **6**: 139-144
13 **Boggino HE,** Fernandez MP, Logroño R. Cytomorphology of gastrointestinal stromal tumor: diagnostic role of aspiration cytology, core biopsy, and immunochemistry. *Diagn Cytopathol* 2000; **23**: 156-160
14 **Liu X**, Ma D, Wu L, Bai C, Hu H. [Expression and clinical significance of c-kit oncogene in gastrointestinal stromal tumors]. *Zhonghua Waike Zazhi* 2002; **40**: 277-279
15 **Hou Y**, Wang J, Zhu X, Du X, Sun M, Zheng A. [A clinicopathologic and immunohistochemical study on 76 cases of gastrointestinal stromal tumors]. *Zhonghua Binglixue Zazhi* 2002; **31**: 20-25
16 **Kim MK**, Lee JK, Park ET, Lee SH, Seol SY, Chung JM, Kang MS, Yoon HK. [Gastrointestinal stromal tumors: clinical, pathologic features and effectiveness of new diagnostic criteria]. *Korean J Gastroenterol* 2004; **43**: 341-348
17 **Okamoto K**, Kato S, Arima N, Fujii T, Morimatsu M, Imaizumi T. Cyclin-dependent kinase inhibitor, p21Waf1, regulates vascular smooth muscle cell hypertrophy. *Hypertens Res* 2004; **27**: 283-291
18 **Pindzola JA,** Palazzo JP, Kovatich AJ, Tuma B, Nobel M. Expression of p21WAF1/CIP1 in soft tissue sarcomas: a comparative immunohistochemical study with p53 and Ki-67. *Pathol Res Pract* 1998; **194**: 685-691
19 **Migaldi M**, Sgambato A, Garagnani L, Ardito R, Ferrari P, De Gaetani C, Cittadini A, Trentini GP. Loss of p21Waf1 expression is a strong predictor of reduced survival in primary superficial bladder cancers. *Clin Cancer Res* 2000; **6**: 3131-3138
20 **van der Woude CJ,** Kleibeuker JH, Tiebosch AT, Homan M, Beuving A, Jansen PL, Moshage H. Diffuse and intestinal type gastric carcinomas differ in their expression of apoptosis related proteins. *J Clin Pathol* 2003; **56**: 699-702
21 **Wheaton S**, Netser J, Guinee D, Rahn M, Perkins S. Bcl-2 and bax protein expression in indolent versus aggressive B-cell non-Hodgkin's lymphomas. *Hum Pathol* 1998; **29**: 820-825
22 **Jansson A,** Sun XF. Bax expression decreases significantly from primary tumor to metastasis in colorectal cancer. *J Clin Oncol* 2002; **20**: 811-816
23 **Chao H**, Sun J, Lu S. [Bax gene expression in endometrial carcinoma]. *Zhonghua Zhongliu Zazhi* 2001; **23**: 214-216
24 **Liu Y**, Chen C, Chen C, Hsieh H, Chang C, Shyu J, Yen C, Harn H. Apoptosis and Fas-ligand expression correlate to the histopathological grade of gastric smooth muscle tumors. *J Surg Res* 2001; **95**: 92-98
25 **Noguchi T,** Sato T, Takeno S, Uchida Y, Kashima K, Yokoyama S, Müller W. Biological analysis of gastrointestinal stromal tumors. *Oncol Rep* 2002; **9**: 1277-1282
26 **Yang F**, Wang W. [Effects of overexpression of p21WAF1/CIP1 on the malignant phenotype and apoptosis of human hepatocellular carcinoma cells]. *Zhonghua Zhongliu Zazhi* 1999; **21**: 99-101
27 **Matsushita H**, Morishita R, Kida I, Aoki M, Hayashi S, Tomita N, Yamamoto K, Moriguchi A, Noda A, Kaneda Y, Higaki J, Ogihara T. Inhibition of growth of human vascular smooth muscle cells by overexpression of p21 gene through induction of apoptosis. *Hypertension* 1998; **31**: 493-498
28 **Rau B**, Sturm I, Lage H, Berger S, Schneider U, Hauptmann S, Wust P, Riess H, Schlag PM, Dörken B, Daniel PT. Dynamic expression profile of p21WAF1/CIP1 and Ki-67 predicts survival in rectal carcinoma treated with preoperative radiochemotherapy. *J Clin Oncol* 2003; **21**: 3391-3401
29 **Gartel AL**, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther* 2002; **1**: 639-649

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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BASIC RESEARCH

Formation of microchimerism in rat small bowel transplantation by splenocyte infusion

Da-Xun Piao, Tao Jiang, Lian-Xin Liu, An-Long Zhu, Shao-Feng Jin, Ying-Hui Guan

Da-Xun Piao, Tao Jiang, Lian-Xin Liu, An-Long Zhu, Department of General Surgery, the First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Shao-Feng Jin, Department of General Surgery, the Affiliated Hospital of Harbin Institute of Technology, Harbin 150001, Heilongjiang Province, China
Ying-Hui Guan, Department of General Surgery, the Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Supported by grant from Program for Innovative Ability of Key Teachers in Universities of Heilongjiang Province
Correspondence to: Dr. Lian-Xin Liu, Department of General Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China. liulianxin@medmail.com.cn
Telephone: +86-451-53643849-5885 Fax: +86-451-53670428
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CONCLUSION: Donor splenocyte infusion combined with CsA decreases remarkably the rejection and prolongs the survival time after rat small bowel transplantation.

Key words: Microchimerism; Splenocyte infusion; Immunologic tolerance; Small bowel transplantation

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Abstract

AIM: To investigate the effect of donor splenocyte infusion combined with cyclosporine A (CsA) on rejection of rat small bowel transplantation (SBT).

METHODS: Male Sprague-Dawley (SD) rats and female Wistar rats weighing 230-270 g were used as donors and recipients respectively in the study. Heterotopic small bowel transplantation was performed. The rats were divided into three groups: group one receiving allotransplantation (SD→Wistar), group two receiving allotransplantation (SD→Wistar) + donor splenocyte infusion, group three receiving allotransplantation (SD →Wistar) + donor splenocyte infusion + CsA followed by CsA 10 mg/kg per day after transplantation, in which recipient Wistar rats were injected with 2 ×10^8 SD splenocytes 28 d before transplantation, and treated with CsA after transplantation. Finally, the specific DNA fragment of donor Y chromosome was detected in recipient peripheral blood and skin by PCR. The survival time after small bowel transplantation was observed. Gross and histopathological examinations were performed.

RESULTS: The survival time after small bowel transplantation was 7.1 ± 1.2 d in group 1, 18.4 ± 3.6 d in group 2 and 31.5 ± 3.1 d in group 3. The survival time was significant longer (P < 0.01) in group 3 than in groups 1 and 2. The gross and histopathological examination showed that the rejection degree in group 3 was lower than that in groups 1 and 2.

INTRODUCTION

Small bowel transplantation (SBT) has become an accepted therapy for intestinal diseases in patients who require total parenteral nutrition[1,2]. Because of the rich lymphatic tissue in small bowel and its mesentery, the mesenteric lymph nodes and lymphatic plexus are transplanted along with the small bowel. Thus, small bowel transplantation has a more severe immune rejection compared with other organ transplantations[3]. Immune rejection is the leading cause of failure in small bowel transplantation[4-7]. Although the results of SBT have been dramatically improved during the past few years, the major impediment to success in SBT is still acute rejection (AR). The key steps toward a successful transplantation are therefore to attenuate immune reactions and to induce immune tolerance to grafts.

The spleen is an immunologically privileged organ. The incidence rate and degree of rejection after spleen transplantation are much lower than those of other solid organ transplantations. Splenocyte chimerism has been successful in inducing tolerance in acute and chronic rejection liver transplant models[9]. Both in experimental study and in clinical practice, splenocytes (including lymphocytes, dendritic cells, Kupffer cells, *etc*) play an important role in immune tolerance induction. Although splenocyte chimerism can effectively decline the immune reactions in organ transplantation, but whether the administration of spleen cells to recipients has the same effect in SBT is uncertain. Some parameters were tested to confirm the anti-rejection effect of splenocyte infusion

combined with CsA on rat small bowel transplantation in this study.

MATERIALS AND METHODS

Animal preparation

Healthy inbred male Sprague-Dawley (SD) rats as donors, and inbred female Wistar rats as recipients, weighing 250 ± 20 g, provided by Medical Experiment Animal Center, Harbin Medical University, were housed in standard animal facilities, and fed with commercially available rat chow and tap water *ad libitum* for 1 wk before test to acclimatize to the laboratory. The donor and recipient were paired according to the similar body weight.

Rat small bowel transplantation

All procedures were performed under inhalation anesthesia with ether. The entire small bowel from the ligament of Treitz to the ileocecal valve was isolated with the superior mesenteric artery on a segment of aorta and portal vein[14]. After donor systemic heparinization (300 U), the graft was perfused with 20 mL of cold lactated Ringer's solution *via* the aorta. The lumen was also washed in 20 mL of the same solution. In the recipient, end-to-side vascular anastomoses were performed between the graft aorta and recipient infra-renal aorta and between the graft portal vein and recipient inferior vena cava with 10-0 sutures using the standard microsurgical technique. The superior extremity of the transplanted small bowel was ligated and a distal small bowel stoma was performed on the left abdominal wall. Animals that died within 3 d were considered as technical failures and excluded from data collection[10-12].

Spleen cell preparation

SD rats were sacrificed by decapitation. Spleens were collected and kept on ice in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). The spleens were disrupted in the medium by pressing spleen fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times with RPMI 1640 medium. Viable nucleated cells were counted and adjusted usually to 2.0×10^7/mL.

Experimental groups and postoperative care

The rats were divided into three groups: group 1 as allotransplantation group (SD → Wistar, $n = 10$), group2 as allotransplantation (SD → Wistar) + donor splenocyte infusion, group 3 as allotransplantation (SD → Wistar) + donor splenocyte infusion + CsA, with CsA 10 mg/kg per day after transplantation, in which recipient Wistar rats were injected with 2×10^8 SD splenocytes 28 d before transplantation and treated with CsA after transplantation. Animals were fed with only sugar water (7 g/d) on d 1, rat chow and water on d 2 and thereafter. The rats' mental state, appetite, and ejection liquid of small bowel stoma were also observed.

Analysis of microchimerism

Microchimerism in peripheral blood and skin of Wistar rat recipients was assessed by PCR. Blood and skin were collected, and genomic DNA was isolated from buffy coat with a DNA extract kit and from skin by the proteinase k-phenol-chloroform method. Microchimerism was detected by specific primers for the donor Y chromosome. PCR was performed with 100 ng of the DNA template, 18 pmol of forward (5'- CGT GGA GAG CGC AAG TT -3', p1) and reverse primers (5'- GTC GCT GTT TCT GCT GTA GTT A -3', p2). The primers were designed to distinguish donor DNA from recipient DNA, and yielded a visible PCR product of 154 bp with the donor DNA template under ethidium bromide fluorescence. The 50 μL reaction contained 10 mmol/L Tris (pH 8.3), 50 mmol/L KCL, 1.5 mmol/L $MgCl_2$, 0.001% gelatin (Perkin- Elmer, Foster City, CA), 50 g/L BSA, 0.2 mmol/L dNTPs, and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer). The cycling conditions were: at 94℃ for 4 min, followed by 35 cycles at 94℃ for 60 s, at 55℃ for 60 s, at 72℃ for 60 s and a final extension at 72℃ for 8 min in a GeneAmp PCR System 2400 (Perkin Elmer). The products were separated on 1.5% agarose gel containing ethidium bromide.

Histopathological analyses

Rats' small bowel allografts were excised from stoma or by laparotomy and fixed in 10% formalin. The fixed tissue was paraffin embedded, and tissue sections were stained with hematoxylin and eosin (HE). Rejection was graded histologically according to the phase of acute intestinal rejection established by Rosemurgy *et al*[13] and Sudan *et al*[14]. The sections were graded for tissue injury using a scale of 0 (none) to 7 (severe) based on the following criteria: 0: normal mucosa; 1: development of subepithelial (Gruenhagen's) spaces at villus tips; 2: extension of the subepithelial space with moderate epithelial cell lifting from the lamina propria; 3: massive lifting down sides of villi, some denuded tips; 4: denuded villi, dilated capillaries; 5: disintegration of the lamina propria; 6: crypt layer injury; 7: transmucosal infarction. All histological analyses were performed in a blinded fashion to avoid bias.

Graft survival

All recipients were followed up by visual inspection and submitted to autopsy soon after they died. Graft survival time was defined as the time until death of recipient due to immune rejection.

Statistical analysis

All data were analyzed by Student's *t* test and expressed as mean ± SD. $P < 0.05$ was considered significant and $P < 0.01$ very significant.

RESULTS

Gross observation

The rats awaked soon after operation. In allotransplantation group (group 1), the rats presented various degrees of lethargy, anorexia hair disorder, unresponsiveness to outside stimulation and body weight loss. Intestinal graft was pale. Intestinal lumina was enlarged with massive adhesion and gradually aggravated, accompanying mass purulent discharge. Intestinal perforation occurred in some severe cases. In allotransplantation + donor splenocyte

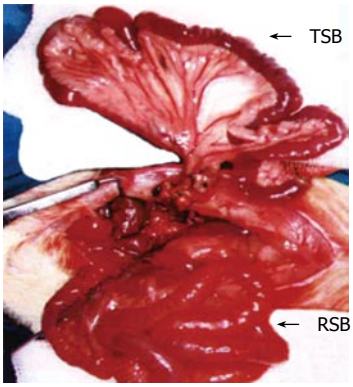

Figure 1 Heterotopic small bowel transplantation in rats. The graft vasculature was anastomosed. TSB: transplanted small bowel; RSB: recipient's small bowel.

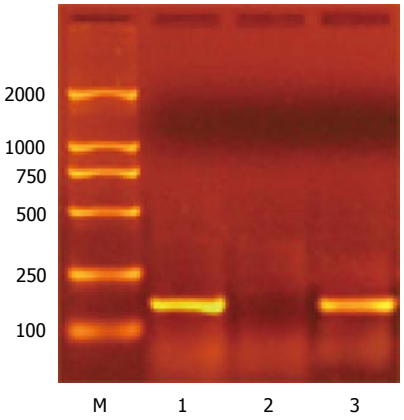

Figure 2 Agarose gel electrophoresis of PCR products. M: 2 Kb DNA ladder; lane 1: PCR amplification bands from normal SD rats; lane 2: PCR amplification bands from allotransplantation group rats; lane 3: PCR amplification bands from allotransplantation + splenocyte infusion group rats.

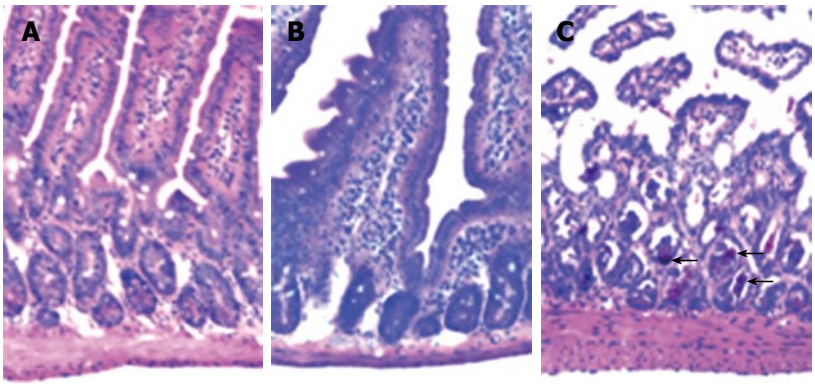

Figure 3 Histopathological findings in different groups after SBT. **A**: Few histopathological changes in the normal tissue (group 3); **B**: Intact villi and some infiltrates in allografts obtained from donor splenocyte recipients (group 2); **C**: Inflammatory infiltrates throughout the intestine and extensive apoptosis (arrows) and ulceration in the transplanted intestine (group 1).

Table 1 Survival time after small bowel transplantation in rats (mean ± SD)

Group	*n*	Survival time (d)
group 1	10	7.1 ± 1.2
group 2	10	18.4 ± 3.6
group 3	10	31.5 ± 3.1[b]

[b]$P < 0.01$ *vs* groups 1 and 2.

Table 2 Histologic grading of graft after small bowel transplantation in rats (mean ± SD)

Group	*n*	Histologic grading
group 1	10	6.4 ± 2.81
group 2	10	5.1 ± 1.06
group 3	10	3.2 ± 1.67[a]

[a]$P < 0.05$ *vs* groups 1 and 2.

infusion group (group 2), the rats were vigorous, sensitive to outside stimulation, and low-grade adhesion occurred 7 d after transplantation. In allotransplantation + donor splenocyte infusion + CsA group (Group 3), the rats were vigorous, and sensitive to outside stimulation, and low-grade adhesion occurred 6 d after transplantation (Figure 1).

Donor cell chimerism

Male donor cell chimerisms in recipients were determined by analyzing metaphase spreads for the presence of donor Y chromosomes. Y chromosomes were found in rats of the spleen cell infusion group but not in rats of allotransplantation group (Figure 2).

Survival time after transplantation

The survival time after small bowel transplantation was 7.1 ± 1.2 d in group 1, 18.4 ± 3.6 d in group 2, and 31.5 ± 3.1 d in group 3. The survival time was significant longer ($P < 0.01$) in group 3 than in groups 1 and 2 (Table 1).

Histopathologic examination

Few histopathological changes and inflammatory infiltrate were detected in rats of group 3 (Figure 3A). After allotransplantation + splenocyte infusion treatment, some histopathological features of rejection were found, villi were intact but showed some blunting, crypts had no necrosis and lymphocyte infiltrate was minimal. A significant decrease in AR changes was observed in grafts of recipients treated with donor splenocyte infusion (Figure 3B). In group 1, severe rejection was characterized by complete villus flatting, epithelial apoptosis, and transmural cellular infiltrate (Figure 3C, Table 2).

DISCUSSION

In clinical practice, immune rejection induced by organ transplants necessitates the use of potent immunosuppressive drugs. However, excessive dosage of immunosuppressive agents may result in severe side effects, such

as hypertension, hepatic and/or renal toxicity. Moreover, prolonged usage of immunosuppressants often leads to severe infection and increased susceptibility to malignant tumors, thus critically affecting the health of recipients. It is therefore imperative to assess the protective effect of immune tolerance on organ transplantation. Recently, it has been reported that donor-derived multilineage hemopoietic cell microchimerism is a prerequisite for tolerance induction in organ allograft recipients[15,16]. In an effort to augment the natural microchimerism that occurs following organ transplantation, adjunctive perioperative donor spleen cell infusion should be considered in conventionally immunosuppressed human organ transplant recipients[17,18]. It was reported that donor spleen cell infusion can enhance liver allograft survival in humans[19] because of the lack of recipient type antigen presenting cells (APC)[20]. In our study, the survival time of the rats that received infusion of donor spleen cells combined with CsA was significantly longer compared to those that did not receive it.

The spleen is an immunologically privileged organ. The rejection incidence rate and degree of spleen transplantation are much lower than those of other solid organs. The spleen cell infusion can induce donor-specific transplantation tolerance and transient microchimerism. In our study, SD splenocyte chimerism was established in Wistar rats. SB grafts remained normal and were well vascularized. By contrast, the bowel of control animals showed AR.

The survival time was significant longer (31.5 ± 3.1 d *vs* 7.1 ± 1.2 d, $P < 0.01$) in group 3 than groups 1 and 2. The rejection degree in group 3 was lower than that in group 1. Rats in group 3 had better histological structures than rats in group 1 (3.2 ± 1.67 *vs* 6.4 ± 2.81, $P < 0.05$), suggesting that donor spleen cell transfusion combined with cyclosporine A is a simple and practical method to suppress rejection of small bowel transplantation in clinical practice.

In conclusion, donor-specific tolerance induced by splenocyte chimerism prevents AR in an experimental model of highly immunogenic SB allografts. Central tolerance through splenocyte chimerism can be used to study the immune tolerance mechanisms. Further work is needed to reveal if chimerism is induced by spleen cell transfusion.

REFERENCES

1 **Asfar S**, Zhong R, Grant D. Small bowel transplantation. *Surg Clin North Am* 1994; **74**: 1197-1210
2 **Platell CF**, Coster J, McCauley RD, Hall JC. The management of patients with the short bowel syndrome. *World J Gastroenterol* 2002; **8**: 13-20
3 **Qian YB**, Cheng GH, Huang JF. Multivariate regression analysis on early mortality after orthotopic liver transplantation. *World J Gastroenterol* 2002; **8**: 128-130
4 **Nishida S**, Levi D, Kato T, Nery JR, Mittal N, Hadjis N, Madariaga J, Tzakis AG. Ninety-five cases of intestinal transplantation at the University of Miami. *J Gastrointest Surg* 2002; **6**: 233-239
5 **Kato T**, Ruiz P, Thompson JF, Eskind LB, Weppler D, Khan FA, Pinna AD, Nery JR, Tzakis AG. Intestinal and multivisceral transplantation. *World J Surg* 2002; **26**: 226-237
6 **Cicalese L**, Rastellini C, Sileri P, Abcarian H, Benedetti E. Segmental living related small bowel transplantation in adults. *J Gastrointest Surg* 2001; **5**: 168-172; discussion 173
7 **Reyes J**. Intestinal transplantation for children with short bowel syndrome. *Semin Pediatr Surg* 2001; **10**: 99-104
8 **Nakano Y,** Monden M, Valdivia LA, Gotoh M, Tono T, Mori T. Permanent acceptance of liver allografts by intraportal injection of donor spleen cells in rats. *Surgery* 1992; **111**: 668-676
9 **Bramhall SR**, Minford E, Gunson B, Buckels JA. Liver transplantation in the UK. *World J Gastroenterol* 2001; **7**: 602-611
10 **Nakao A**, Tahara K, Inoue S, Tanaka N, Kobayashi E. Experimental models of small intestinal transplantation in rats: orthotopic versus heterotopic model. *Acta Med Okayama* 2002; **56**: 69-74
11 **Motohashi H**, Masuda S, Katsura T, Saito H, Sakamoto S, Uemoto S, Tanaka K, Inui KI. Expression of peptide transporter following intestinal transplantation in the rat. *J Surg Res* 2001; **99**: 294-300
12 **Li YX**, Li JS, Li N. Improved technique of vascular anastomosis for small intestinal transplantation in rats. *World J Gastroenterol* 2000; **6**: 259-262
13 **Rosemurgy AS**, Schraut WH. Small bowel allografts. Sequence of histologic changes in acute and chronic rejection. *Am J Surg* 1986; **151**: 470-475
14 **Sudan DL**, Kaufman SS, Shaw BW Jr, Fox IJ, McCashland TM, Schafer DF, Radio SJ, Hinrichs SH, Vanderhoof JA, Langnas AN. Isolated intestinal transplantation for intestinal failure. *Am J Gastroenterol* 2000; **95**: 1506-1515
15 **Hoppe H**, Gasser M, Gassel AM, Vowinkel T, Timmermann W, Otto C, Tykal K, Thiede A. Noninvasive videomicroscopic monitoring of rat small-bowel rejection. *Microsurgery* 1999; **19**: 89-94
16 **Starzl TE**, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. *Lancet* 1992; **339**: 1579-1582
17 **Starzl TE**, Demetris AJ, Trucco M, Murase N, Ricordi C, Ildstad S, Ramos H, Todo S, Tzakis A, Fung JJ. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. *Hepatology* 1993; **17**: 1127-1152
18 **Fontes P**, Rao AS, Demetris AJ, Zeevi A, Trucco M, Carroll P, Rybka W, Rudert WA, Ricordi C, Dodson F. Bone marrow augmentation of donor-cell chimerism in kidney, liver, heart, and pancreas islet transplantation. *Lancet* 1994; **344**: 151-155
19 **Shapiro R**, Rao AS, Fontes P, Jordan M, Scantlebury VP, Vivas C, Demetris AJ, Zeevi A, Rybka W, Carroll P. Combined kidney/bone marrow transplantation--evidence of augmentation of chimerism. *Transplantation* 1995; **59**: 306-309
20 **Ricordi C**, Karatzas T, Nery J, Webb M, Selvaggi G, Fernandez L, Khan FA, Ruiz P, Schiff E, Olson L, Fernandez H, Bean J, Esquenazi V, Miller J, Tzakis AG. High-dose donor bone marrow infusions to enhance allograft survival: the effect of timing. *Transplantation* 1997; **63**: 7-11

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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CLINICAL RESEARCH

Hepaticojejunostomy for hepatolithiasis: A critical appraisal

Shao-Qiang Li, Li-Jian Liang, Bao-Gang Peng, Jia-Ming Lai, Ming-De Lu, Dong-Ming Li

Shao-Qiang Li, Li-Jian Liang, Bao-Gang Peng, Jia-Ming Lai, Ming-De Lu, Dong-Ming Li, Department of Hepatobiliary Surgery, the First Affiliated Hospital of Sun Yet-san University, Guangzhou 510080, Guangdong Province, China
Correspondence to: Professor Li-Jian Liang, MD, Department of Hepatobiliary Surgery, the First Affiliated Hospital of Sun Yet-san University, Guangzhou 510080, Guangdong Province, China. lianglj@medmail.com.cn
Telephone: +86-20-87755766-8096 Fax: +86-20-87755766-8663
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Abstract

AIM: To evaluate the long-term outcome and surgical indications of hepaticojejunostomy (HJ) for the treatment of hepatolithiasis.

METHODS: Three hundred and fourteen elective cases with hepatolithiasis but without biliary stricture or cystic dilatation treated in the past 10 years were reviewed retrospectively. The patients were divided into HJ group and T tube drainage group according to biliary drainage procedure. Furthermore, four subgroups were subdivided by hepatectomy as a balance factor, group A_1: hepatectomy+HJ; group A_2: choledochoctomy+HJ; group B_1: hepatectomy + choledochoctomy T tube drainage; group B_2: choledochoctomy + T tube drainage. The stone residual rate, surgical efficacy and long-term outcome were compared among different procedures.

RESULTS: There was no surgical mortality among all patients. The total hospital mortality was 1.6%. The overall stone residual rate after surgical clearance was 25.9%. There was no statistical difference between HJ group and T tube drainage group in terms of stone residual rate after surgical clearance, however, after postoperative choledochoscopic lithotripsy, the total stone residual rate of T tube drainage group was significantly lower than that of HJ group (0.5% *vs* 16.7%, $P < 0.01$). Hepatectomy + choledochoctomy tube drainage achieved the optimal therapeutic effect, only 8.2% patients suffered from an attack of cholangitis postoperatively, which was significantly lower than that of hepatectomy + HJ (8.2% *vs* 22.0%, $P = 0.034$). The major reason for postoperative cholangitis was stone residual in the HJ group (16/23, 70.0%), and stone recurrence in the T tube drainage group (34/35, 97.1%). The operative times were significantly prolonged in those undergoing HJ, and the operative morbidity of HJ was higher than those of T tube drainage.

CONCLUSION: The treatment result of HJ for hepatolithiasis is not satisfactory in this retrospective study due to high rate of stone residual and postoperative cholangitis. HJ could not drain residual stone effectively. HJ may hinder post-operative choledochoscopic lithotripsy, which is the optimal management for postoperative residual stone. The indications of HJ for hepatolithiasis should be strictly selected.

Key words: Hepatolithiasis; Hepaticojejunostomy; Outcome

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INTRODUCTION

Hepatolithiasis is a common disease in Southeast Asia and is especially prevalent in China[1,2]. This disease features a high stone residual and recurrence and its long-term outcome is far from satisfactory. The principles for the treatment of hepatolithiasis are complete clearance of stones, eradication of the diseased bile duct (stricture) and atrophic liver tissue and reconstruction of satisfactory bile drainage. Biliary-enteric anastomosis which mostly includes choledochoduodenostomy (CD) and Roux-en-Y hepaticojejunostomy(HJ), is one of the most common procedures used for hepatolithiasis. Due to the sump syndrome and high risk of stasis following CD[3,4], many authors prefer HJ to CD as the standard procedure for benign biliary diseases[4-6]. In fact, we have used HJ instead of CD to reconstruct enteric biliary drainage for hepatolithiasis or bile duct stricture in our department since 1990.

In the cases of hepatolithiasis simultaneously with extrahepatic bile duct stricture or congenital cyst dilatation, because the diseased bile duct should be resected, HJ is the treatment of choice to reconstruct bile drainage. However, in the cases without bile duct stricture or cystic dilatation, whether HJ is suitable remains controversial[7-9].

In the current study, we reviewed the cases with hepatolithiasis treated surgically in our department in the past 10 years retrospectively, and evaluated the outcome and surgical indications of HJ for the treatment of hepatolithiasis.

MATERIALS AND METHODS

Patients

A total of 425 consecutive patients with hepatolithiasis treated surgically at the Department of Hepaticobiliary Surgery, the First Affiliated Hospital, Sun Yet-san University from June 1992 to June 2002 were reviewed retrospectively. According to our study purpose, the following cases were excluded because of no alternative surgical procedures for their conditions at that time, respectively, (1) 21 cases of hepatolithiasis complicated with acute obstructive suppurative cholangitis, who underwent choledochoctomy, stone extraction and T tube drainage emergently once the diagnosis was confirmed; (2) 21 cases of hepatolithiasis with congenital choledochal cyst or Caroli's disease, who underwent stone removal, cyst resection or hepatectomy and HJ; (3) 23 cases of hepatolithiasis with left or right hepatic ducts or their second branches stricture, who received diseased bile duct resection and (or) bile duct stricturoplasty and HJ; (4) 46 cases of hepatolithiasis complicated with cholangiocarcinoma were also excluded. The remaining 314 elective cases were enrolled in this study, including 122 men, and 192 women. The mean age of whole group was 48 years (range: 15-88).

Grouping

According to the different procedures of bile drainage, 314 patients were divided into HJ group (n = 123) and T tube drainage group (n = 191). Because some cases in these two groups underwent hepatectomy simultaneously, we used hepatectomy as a balance factor to subdivide these two groups into four subgroups, Group A_1: Hepatectomy + HJ (n = 76); group A_2: choledochoctomy + HJ (n = 47); group B_1: hepatectomy + choledochoctomy T tube drainage (n = 85); group B_2: choledochoctomy + T tube drainage(n = 106). The rate of residual stone, operative complications and therapeutic outcomes were compared among these groups.

Procedure of HJ

The standard procedure of HJ was an end-to-side, mucosa-to-mucosa anastomosis of the intra- and (or) extrahepatic duct with a Roux-en-Y jejunal loop measuring 40 to 60 cm in length. The anastomosis was sutured with absorbable material (Vircyl, 4-0, Johnson Ltd, USA) interruptedly. A rubber tube measuring 3.5-5 mm in diameter was placed through biliary-enteric anastomosis retrogradely for later cholangiography or choledochoscopic manipulation. This drainage tube was removed when no stone resided within the biliary tract proofed by a cholangiogram at postoperative d 14. If there was residual stone proofed by cholangiography, the drainage tube tract was left for 6 wk to allow subsequent choledochoscopic manipulation. The indications of HJ we used previously were (1) the diameter of the common bile duct was larger than 2 cm; (2) intrahepatic stone located bilaterally; (3) peripheral bile duct stone that could not be cleared during surgery.

Measurement of residual stone

The diagnosis of postoperative stone residual was based on the cholangiogram performed through the T tube and transanastomotic drainage tube at postoperative d 14 or ultrasound findings. Once residual stone was found, choledochoscopic stone extraction or lithotripsy (four-direction fiber choledochoscopy, 3.5 mm in diameter, Olympus Co., Japan) was done 6 wk after operation. This was repeated at 1 wk interval until the residual stone was completely cleared, or as clear as possible.

Postoperative follow-up

Follow-up was performed by reviewing medical records and patient interviews or telephone interview. At the end of this study, totally 241 out of 309 completed the follow-up (period: 2-12 years, median 7.6), the other patients were lost. Cholangitis was considered when patient presented with right upper quadrant pain, chill, fever or jaundice.

Statistical analysis

Patients' database was established by SPSS 11.0 software. t-test, rank test (continuous data) and Fisher's exact test, Chi-square test (categorical data) were used. $P < 0.05$ was considered statistical difference.

RESULTS

Operative mortality and hospital death

There was no surgical mortality in this study. Five cases (1.6%) died of hepatic failure postoperatively during their hospital stay, including 1 case in subgroup A_1, 2 cases in subgroup A_2, and 2 cases in subgroup B_2.

Patients' clinical characteristics

Patients' demographic and clinical data are shown in Table 1. The number of patients who had previous bile surgery of subgroups A_1 and A_2 was greater than those of subgroups B_1 and B_2, respectively ($P < 0.05$). The history and operating times of patients in the subgroups A_1 and A_2 were longer than those in subgroups B_1 and B_2, respectively ($P < 0.05$); the amount of blood loss in subgroups A_1 and A_2 was larger than those in the subgroups B_1 and B_2 respectively ($P < 0.05$).

Postoperative stone residual

The diagnosis of postoperative stone residual was based on the cholangiogram and ultrasound findings postoperatively. The most common site of stone residual was the intrahepatic duct. The overall stone residual rate after surgical clearance in our series was 25.9% (Table 2). There was no significant difference of stone residual between the HJ group and the T tube drainage group (20.3% *vs* 28.3%, $P > 0.05$). The cases who underwent hepatectomy [group ($A_1 + B_1$)] had less stone residual than those who did not, [group ($A_2 + B_2$)] ($P < 0.05$).

Postoperative complications

The common postoperative complications are listed in Table 3. The complications in subgroup A_1 was more than that in subgroup A_2 ($\chi^2 = 4.324$, $P < 0.05$).

Management of residual stone

The treatment of choice for postoperative residual stone

Table 1 Patients' demographic and clinical data

Groups	*n*	PS[1] *n* (%)	Age (yr)	Hospital stay (d)	Operative time (min)	History[2] (yr)	Bleeding[2] (mL)
HJ group	123	90 (73.17)					
+hepatectomy (A1)	76	55 (72.37)[a]	45.4 ± 13.8	32.1 ± 12.0	282.9 ± 99.3[a]	8.5[a] (0.1-40)	500.0[a] (150-27 000)
left lateral segmentectomy	52						
left hepatectomy	15						
right hepatectomy	3						
segmentectomy	6						
+CBD exploration (A2)	47	35 (74.47)[c]	51.8 ± 13.5	27.0 ± 8.5	226.1 ± 80.4	8.0[c] (0.1-35)	300.0 (100-3000)
T tube drainage group	191	102 (53.40)					
+hepatectomy (B1)	85	46 (54.12)	46.6 ± 12.6	29.2 ± 10.6	189.4 ± 54.1[c]	3.5 (0.1-40)	300.0[c] (100-5000)
left lateral segmentectomy	57						
left hepatectomy	13						
right hepatectomy	4						
segmentectomy	11						
+ CBD exploration (B2)	106	56 (52.83)	49.8 ± 15.7	28.3 ± 16.6	166.4 ± 97.7	3.0 (0.1-40)	150.0 (30-18 000)

[a]$P < 0.05$ *vs* group B1, [c]$P < 0.05$ *vs* group B2. [1]ps: previous bile surgery, [2]expressed as median (range). Age, hospital stay and operative time were expressed as mean ± SD. CBD: common bile duct; HJ: hepaticojejunostomy.

Table 2 Sites and rates of residual stone

Groups	*n*	Site of stone residual					Rate
		Left liver	Right liver	CBD	Left liver	Right liver	Total (%)
HJ group	123	8	14	1	2	25	20.3[a]
+hepatectomy (A1)	76	3	10	1	0	14	18.4
+CBD exploration (A2)	47	5	4	0	2	11	23.4
T tube drainage group	191	17	22	3	12	54	28.3
+ hepatectomy (B1)	85	5	10	1	2	18	21.2
+ CBD exploration (B2)	106	12	12	2	10	36	34.0

HJ, hepaticojejunostomy; CBD, common bile duct. [a]$P > 0.05$ *vs* T tube drainage group.

Table 3 Postoperative complications

Complications	Group A1 (*n* = 76)	Group A2 (*n* = 47)	Group B1 (*n* = 85)	Group B2 (*n* = 106)
Wound infection	6	3	5	7
Subphrenic collection/infection	7	0	3	0
Pleural infusion	3	0	2	0
Bile leakage	2	0	3	1
Biliary hemorrhage	1	0	0	2
Liver abscess	1	0	0	0
Liver failure	1	2	0	6
Pulmonary infection	0	1	1	1
Septicemia	1	0	0	0
Total	22	6	14	17
%	28.95[a]	12.77	16.47	16.04

[a]$P < 0.05$ *vs* group A2.

Table 4 Follow-up and postoperative cholangitis

Groups	*n*	Follow-up patients (*n*, %)	Postoperative cholangitis (*n*, %)	Causes of cholangitis ST	SR	SRE	RC
HJ group	123	96 (78.0)	23 (24.0)	3	16	(3)[4]	1
+ hepatectomy (A1)	76	59 (77.6)	13 (22.0)	1	9	3	0
+ CBD exploration (A2)	47	37 (78.7)	10 (27.0)	2	7	(2)[3]	1
T tube drainage group	191	145 (76.0)	35 (24.1)	0	1	34	0
+ hepatectomy (B1)	85	61 (71.8)	5 (8.2)[1]	0	0	5	0
+ CBD exploration (B2)	106	84 (79.2)	30 (35.7)[2]	0	1	29	0

[1]$P = 0.034$ *vs* group A1. [2]$P = 0.349$ *vs* group A2. [3,4]these two (or three patients) patients who had stone recurrence also had stricture of anastomosis. HJ: hepaticojejunostomy, ST: stricture of anastomosis or intrahepatic bile duct, SR: stone residual; SRE: stone recurrence; RC: reflux cholangitis.

was choledochoscopic lithotripsy through the T tube or the transanastomotic drainage tube route 6 wk after operation. Totally 54 cases had residual stones in the T tube drainage group, and 53 had their stones completely cleared by choledochoscopic lithotripsy after 1 to 6 sections. Only 1 case was failed because his T tube withdrew simultaneously after discharge. However, only 5 cases whose residual stones could be successfully eliminated in HJ group, the other 20 cases failed because their fistula were unsuitable or difficult for the entry of choledochoscope, or the long Roux-en-Y loop hindered the technical manipulation. The overall stone residual rate of the T tube drainage group was significantly lower than the HJ group after choledochoscopic lithotripsy [0.5% (1/189) *vs* 16.7% (20/120), $P < 0.01$].

Postoperative cholangitis

Postoperative cholangitis presented by right upper quadrant pain, chill, fever and jaundice occurred at least once in 58 cases (Table 4). Five cases in the HJ group suffered from 4-6 episodes of cholangitis till the second operations were done. The main causes of cholangitis observed in our series were stricture of the biliary-enteric anastomosis ($n = 3$), stone residual ($n = 16$) or reflux of intestinal fluid ($n = 1$) in the HJ group, and stone recurrence ($n = 34$) or later development of bile stricture for chronic inflammation of bile duct in the T tube drainage group (Table 4).

DISCUSSION

Postoperative stone residual and recurrence still remain a

challenge in the treatment of hepatolithiasis. Though the systemic approaches have been used, the stone residual rate was over 30% in a nationwide survey of 4197 surgical hepatolithiasis cases in China[2]. The overall stone residual rate after surgical clearance in our series was 25.9%. There was no significant difference between the HJ group and the T tube drainage group after surgical clearance. However, when hepatectomy as one of the treatments of choice was considered as a balance factor, we found that among patients who received hepatectomy the stone residual rate was significantly lower than those who did not. This finding is consistent with our previous report[10] and other reports[11-13].

Hepatolithiasis will cause bile duct stricture, liver parenchyma atrophy and chronic fibrosis due to repeated pyogenic cholangitis. These pathologic changes, especially bile duct stricture, may hinder stone extraction during surgery, even though the choledochoscopy was used. Hepatectomy provided the best therapeutic effect for completely removing the stone, the diseased bile duct (stricture ring) and atrophic liver parenchyma as well.

Postoperative choledochoscopic lithotripsy through the T tube or the bile duct drainage tube route is a potential remedial treatment for residual stone, as indicated by the T tube drainage group in this study. However, only 5 cases whose residual stone could be successfully removed by this method in the HJ group. Other cases with residual stone could not be removed due to the difficulty for choledochoscope to access the bile duct through a long (40-60 cm) Roux-Y loop or because the tunnel was too small to enter for the choledochoscope. After postoperative choledochoscopic lithotripsy, the overall stone residual in the T tube drainage group was markedly lower than that in the HJ group (0.5% *vs* 16.7%, $P < 0.01$). This indicates that HJ hinders postoperative choledochoscopic manipulation. For easy access to the biliary tract, hepaticocutaneous jejunostomy with a stoma after biliary surgery is recommended for the convenience of long-term treatment of stone residual or recurrence[14]. However, hepaticocutaneous jejunostomy is not advocated for its possible complications related to fistula, infection, parastomal hernia, early stoma closure, and prolonged surgical procedure or hospitalization[15].

Postoperative cholangitis is a critical factor to evaluate the long-term surgical result of this disease. Hwang *et al*[16] reported that high risk factors related to cholangitis after initial surgery was bile duct stricture, residual stones, recurrent stones, and patients who were treated with nonhepatic resection. Our long-term follow-up (2-12 years, median 7.6) data demonstrated that patients who underwent hepatectomy with CBD exploration and T tube drainage had less attack of cholangitis, and the occurrence of cholangitis was significantly lower than those who underwent hepatecomy with HJ. Though the residual stones were nearly cleared in the T tube drainage group, and the immediate outcome was good, the occurrence of cholangitis was high. This was due to high recurrence of intrahepatic stone secondary to the bile duct stricture which was not treated during the initial surgery. The major reasons for postoperative cholangitis observed in our study were stone residual in the HJ group (16/23, 70.0%), and stone recurrence in the T tube drainage group (34/35, 97.1%).

It was believed that the intrahepatic residual stone located in the peripheral bile duct would drain to Roux-Y loop through a large biliary-enteric anastomotic mouth simultaneously after surgery, therefore, residual stone would be cleared, and furthermore, HJ could prevent biliary-enteric regurgitation[14,17]. To drain a residual stone, HJ is a popular procedure for hepatolithiasis, especially for the case with bilateral stones. Our data showed that the numbers of patients who had previous biliary surgery for hepatolithiasis were higher in the HJ group than that in the T tube drainage group. It suggests that HJ would be the final and optimal procedure for recurred or residual hepatolithiasis at that time. However, many clinical evidences showed that the intrahepatic residual stone could not drain to the Roux-Y loop completely and easily, especially for the stones located at the right posterior lobe and left lateral lobe of the liver. Rather they would accumulate continuously within the bile ducts, which was the main reason for the postoperative cholangitis episodes. This was also evidenced by the long-term follow-up data of the HJ group in this series. Actually, HJ can not completely block biliary-enteric regurgitation. The reflux cholangitis occurring in HJ performed for benign biliary diseases was 10%-15%[18]. Besides the stone residual and bile duct stricture, postoperative cholangitis was also related to the decrease of motility of Roux-en-Y loop itself. Jejunal transaction would lead to abnormal motility of the distal part of jejunum[19,20]. Ducrotte *et al*[21] reported that low motility of the Roux-en-Y loop presented by near-absence of phase Ⅲs activity, and an absence of response to meal was observed in recurrent cholangitis patients, and subsequently, microbes might colonize and overgrow in the loop. Later on they documented a similar result in asymptomatic patients undergoing HJ[22].

HJ permanently eliminates the physiological preventive function of regurgitation by the papilla of Vater, which is a barrier between biliary tract and gastrointestinal tract, and subsequently, enteric biliary reflux and bacterial colonization of the biliary tract may occur[23,24]. Furthermore, in a long-term large series study, Tocchi *et al* found that biliary tract may tend to develop malignancy following biliary-enteric bypass procedures for the benign biliary disease due to chronic inflammation of the bile duct[25]. This indicates that bile duct cancer may be a long-term complication of biliary-enteric drainage.

On the other hand, our data also demonstrated that HJ prolonged the operation time and increased the risk of intraoperative bleeding and blood transfusion.

In conclusion, the overall long-term outcome of HJ for intrahepatic lithiasis was not satisfactory because of its high rate of stone residual and postoperative cholangitis in our study. HJ could not drain the residual stone effectively. Considering the shortcomings of HJ mentioned above, it is of critical importance to consider the surgical indications of HJ for hepatolithiasis. We advocate that HJ is needed only in the following conditions. (1) Hepatolithiasis complicated with extrahepatic ducts or its second branches stricture, which needs stricturoplasty and HJ. (2) Hepatolithiasis with congenital bile duct dilatation in which the dilated bile duct should be resected. (3) Dysfunction of the papilla of Vater, especially in the case of fibrotic

stricture of the papilla of Vater. Our results indicate that if intraheptic stone could not be cleared during surgery, a T tube placement within the CBD rather than HJ would facilitate postoperative choledochoscopic lithotripsy.

REFERENCES

1 **Nakayama F**, Soloway RD, Nakama T, Miyazaki K, Ichimiya H, Sheen PC, Ker CG, Ong GB, Choi TK, Boey J. Hepatolithiasis in East Asia. Retrospective study. *Dig Dis Sci* 1986; **31**: 21-26
2 **Huang ZQ**. [The present status of surgical treatment of intrahepatic lithiasis in a nation-wide survey in China of 4197 operative cases 1981-1985]. *Zhonghua Waike Zazhi* 1988; **26**: 513-522, 572
3 **Parrilla P**, Ramirez P, Sanchez Bueno F, Perez JM, Candel MF, Muelas MS, Robles R. Long-term results of choledochoduodenostomy in the treatment of choledocholithiasis: assessment of 225 cases. *Br J Surg* 1991; **78**: 470-472
4 **Rat P**, Baert D, Arveux P, Martin O, Haas O, Guillard G, Favre JP. Results of bilio-digestive derivative surgical procedures for benign lesions. *Hepatogastroenterology* 1993; **40**: 123-125
5 **Röthlin MA**, Löpfe M, Schlumpf R, Largiadèr F. Long-term results of hepaticojejunostomy for benign lesions of the bile ducts. *Am J Surg* 1998; **175**: 22-26
6 **Tocchi A**, Costa G, Lepre L, Liotta G, Mazzoni G, Sita A. The long-term outcome of hepaticojejunostomy in the treatment of benign bile duct strictures. *Ann Surg* 1996; **224**: 162-167
7 **Chijiiwa K**, Yamashita H, Yoshida J, Kuroki S, Tanaka M. Current management and long-term prognosis of hepatolithiasis. *Arch Surg* 1995; **130**: 194-197
8 **Uchiyama K**, Onishi H, Tani M, Kinoshita H, Ueno M, Yamaue H. Indication and procedure for treatment of hepatolithiasis. *Arch Surg* 2002; **137**: 149-153
9 **Kusano T**, Isa TT, Muto Y, Otsubo M, Yasaka T, Furukawa M. Long-term results of hepaticojejunostomy for hepatolithiasis. *Am Surg* 2001; **67**: 442-446
10 **Liang L**, Huang J, Lu M, Ye W, Peng B, Yin X, Cao X. [Hepatectomy for hepatolithiasis treatment of 354 cases]. *Zhonghua Waike Zazhi* 1998; **36**: 209-211
11 **Fan ST**, Lai EC, Wong J. Hepatic resection for hepatolithiasis. *Arch Surg* 1993; **128**: 1070-1074
12 **Sun WB**, Han BL, Cai JX. The surgical treatment of isolated left-sided hepatolithiasis: a 22-year experience. *Surgery* 2000; **127**: 493-497
13 **Chen DW**, Tung-Ping Poon R, Liu CL, Fan ST, Wong J. Immediate and long-term outcomes of hepatectomy for hepatolithiasis. *Surgery* 2004; **135**: 386-393
14 **Fan ST**, Mok F, Zheng SS, Lai EC, Lo CM, Wong J. Appraisal of hepaticocutaneous jejunostomy in the management of hepatolithiasis. *Am J Surg* 1993; **165**: 332-335
15 **Huang MH**, Chen CH, Yang JC, Yang CC, Yeh YH, Chou DA, Mo LR, Yueh SK, Nien CK. Long-term outcome of percutaneous transhepatic cholangioscopic lithotomy for hepatolithiasis. *Am J Gastroenterol* 2003; **98**: 2655-2662
16 **Hwang JH**, Yoon YB, Kim YT, Cheon JH, Jeong JB. Risk factors for recurrent cholangitis after initial hepatolithiasis treatment. *J Clin Gastroenterol* 2004; **38**: 364-367
17 **Lygidakis N**, Matsakis GN, Tepetes KN, Savanis GS, Touloupakis A, Konstantinidou A, Katsikoyannis J. Intrahepatic cholangiojejunostomy in biliary stricture following resectional liver surgery. *Hepatogastroenterology* 1994; **41**: 1-3
18 **Bismuth H**, Franco D, Corlette MB, Hepp J. Long term results of Roux-en-Y hepaticojejunostomy. *Surg Gynecol Obstet* 1978; **146**: 161-167
19 **Sarna S**, Condon RE, Cowles V. Enteric mechanisms of initiation of migrating myoelectric complexes in dogs. *Gastroenterology* 1983; **84**: 814-822
20 **Kerlin P**, McCafferty GJ, Robinson DW, Theile D. Function of a free jejunal "conduit" graft in the cervical esophagus. *Gastroenterology* 1986; **90**: 1956-1963
21 **Ducrotte P**, Peillon C, Guillemot F, Testart J, Denis P. Could recurrent cholangitis after Roux-en-Y hepaticojejunostomy be explained by motor intestinal anomalies? A manometric study. *Am J Gastroenterol* 1991; **86**: 1255-1258
22 **Le Blanc-Louvry I**, Ducrotté P, Manouvrier JL, Peillon C, Testart J, Denis P. Motility of the Roux-en-Y hepaticojejunostomy in asymptomatic patients. *Am J Gastroenterol* 1999; **94**: 2501-2508
23 **Kusano T**, Masato F, Isa T, Tamai O, Miyazato H, Shiraishi M, Muto Y. Percutaneous transhepatic cholangioscopic lithotripsy and change of biliary manometry patterns. *Hepatogastroenterology* 1999; **46**: 2153-2158
24 **Chuang JH**, Lee SY, Chen WJ, Hsieh CS, Chang NK, Lo SK. Changes in bacterial concentration in the liver correlate with that in the hepaticojejunostomy after bile duct reconstruction: implication in the pathogenesis of postoperative cholangitis. *World J Surg* 2001; **25**: 1512-1518
25 **Tocchi A**, Mazzoni G, Liotta G, Lepre L, Cassini D, Miccini M. Late development of bile duct cancer in patients who had biliary-enteric drainage for benign disease: a follow-up study of more than 1,000 patients. *Ann Surg* 2001; **234**: 210-214

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RAPID COMMUNICATION

Differential treatment and early outcome in the interventional endoscopic management of pancreatic pseudocysts in 27 patients

Uwe Will, Conrad Wegener, Kai-Ivo Graf, Igor Wanzar, Thomas Manger, Frank Meyer

Uwe Will, Conrad Wegener, Kai-Ivo Graf, Igor Wanzar, Department of Gastroenterology, Municipal Hospital, D-07548 Gera, Germany
Thomas Manger, Department of Surgery, Municipal Hospital, D-07548 Gera, Germany
Frank Meyer, Department of Surgery, University Hospital, D-39120 Magdeburg, Germany
Correspondence to: Uwe Will, MD, Department of Gastroenterology, Municipal Hospital, Strasse des Friedens 122, D-07548 Gera, Germany. uwe.will@wkg.srh.de
Telephone: +49-365-8282401 Fax: +49-365-8282402
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Abstract

AIM: Pancreatic pseudocysts (PPC) as a complication of pancreatitis are approached only in the case of abdominal pain, infection, bleeding, and compression onto the gastrointestinal tract or biliary tree.

METHODS: From 02/01/2002 to 05/31/2004, all consecutive patients with symptomatic PPC who underwent an interventional endoscopic approach were evaluated in this pilot case-series study: Group (Gr.) I-Primary percutaneous (external), ultrasound-guided drainage. Gr. II-Primary EUS-guided cystogastrostomy. Gr. III-EUS-guided cystogastrostomy including intracystic necrosectomy.

RESULTS: (="follow up": *n* = 27): Gr. I (*n* = 9; 33.3%): No complaints (*n* = 3); change of an external into an internal drainage (*n* = 4); complications: (a) bleeding (*n* = 1) followed by 3 d at ICU, discharge after 40 d; (b) septic shock (*n* = 1) followed by ICU and several laparotomies for programmed lavage and necrosectomy, death after 74 d. Gr. II (*n* = 13; 48.1%): No complaints (*n* = 11); external drainage (*n* = 2); complications/problems out of the 13 cases: 2nd separate pseudocyst (*n* = 1) with external drainage (since no communication with primary internal drainage); infection of the residual cyst (*n* = 1) + following external drainage; spontaneous PPC perforation (*n* = 1) + following closure of the opening of the cystogastrostomy using clips and subsequently ICU for 2 d. Gr. III (*n* = 5; 18.5%): No complaints in all patients, in average two endoscopic procedures required (range, 2-6).

CONCLUSION: Interventional endoscopic management of pancreatic pseudocysts is a reasonable alternative treatment option with low invasiveness compared to surgery and an acceptable outcome with regard to the complication rate (11.1%) and mortality (3.7%), as shown by these initial study results.

Key words: Pancreatic pseudocyst; Pancreatitis; Interventional endoscopic approach

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INTRODUCTION

Pancreatic pseudocysts (PPC) are complications of acute and chronic pancreatitis[1-4], pancreatic trauma, and pancreatic duct obstruction[4]. For differential diagnosis, PPC needs to be distinguished from cystic tumor lesions of the pancreas. An examination with transabdominal ultrasound and endoscopic ultrasonography (EUS) is considered an initial step of the diagnostic and therapeutic management. PPC are to be treated in the case of subsequent infection, bleeding, and compression onto the gastrointestinal tract or the biliary tree[5]. Abdominal pain is the predominating clinical sign. Fever and septic symptoms are hints for infected PPC. The aim of the study was to demonstrate (1) the differential treatment strategies in PPC[6], (2) the safety of an interventional endoscopic approach of PPC as a reasonable alternative and less invasive treatment option[7,8], and (3) the outcome and short-term follow-up results including problems and complications[7-9].

MATERIALS AND METHODS

From February 01, 2002 to May 31, 2004, all consecutive patients with symptomatic PPC were enrolled in this pilot case-series study. Patients underwent an interventional approach in the case of abdominal pain, fever, increase of C-reactive protein (CrP) above the normal range, and compression onto the gastrointestinal tract or the biliary tree caused by the PPC with

Table 1 Interventional endoscopic approach of PPC: Indications and methodological aspects of the 3 different treatment groups (see also "Patients and methods" section above)

GROUP	Title	*n*	Indication	Method
I	Primary, percutaneous, ultrasound-guided placement of a drainage	9	Septic symptoms & inhomogeneous cystic content with suspected infection of PPC revealed by transabdominal ultrasound & EUS	-Ultrasound-guided puncture of the PPC -Aspiration of 10-20 mL of cystic fluid/content -Laboratory analysis (amylase, lipase, CEA, Ca19-9) -Microbiologic investigation for microbial detection/growth -Placement of a 10-Fr. pigtail drainage (Endoflex, Voerde, Germany) -Rinsing with 20-50 mL NaCl/h (bolus) *via* the drainage -Administration of antibiotics (ceftriaxone [Rocephin®, Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany] plus metronidazol [Clont®, Bayer Vital GmbH, Leverkusen, Germany]) over 7 d
II	Primary, EUS-guided cystogastrostomy	13	Echo-free PPC with no inner echos & no evidence of infection	-Puncture of the cyst with 19-G needle (Wilson-Cook [Cook Deutschland GmbH], Mönchengladbach, Germany) -Cytologic investigation of cystic fluid/content -Placement of a 8.5-Fr.-double pigtail catheter Endoflex, Voerde, Germany) -Administration of antibiotics (ceftriaxone [Rocephin®, Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany]) over 3 d
III	EUS-guided cystogastrostomy with following necrosectomy	5	-Suspected sequester & necrosis within the PPC revealed by ultrasound -Persisting fever & septic symptoms after cystogastrostomy & EUS-guided placement of a drainage	-Introduction of a 0.035-Inch guide wire (MTW Endoskopie, Wesel, Germany) *via* needle in place -Opening of the PPC with needle knife (Erbe Elektromedizin GmbH, Leipzig, Germany) -Enlargement of the PPC opening with balloon dilatation (Boston Scientific Medizintechnik GmbH, Ratingen, Germany) -Endoscopy of the cystic cavity -Removal of the necroses with loop & Dormia´s basket (MTW Endoskopie, Wesel, Germany, each) (min. 2x) -Microbiologic investigation for microbial detection/growth -Placement of a transgastrocystic 8.5-Fr.-double pigtail drainage (Endoflex, Voerde, Germany) -Removal of the pigtail catheter after 3-6 mo -Administration of antibiotics (ceftriaxone plus metronidazol) over 7 d

disturbance(s) of the gastrointestinal passage. Patients were subdivided into 3 groups as shown in Table 1.

A consent form was obtained from each patient enrolled in the study.

RESULTS

Overall, 27 consecutive patients with symptomatic PPC were enrolled in the study over the 28-mo study period. The following details comprise the outcome, subsequent therapeutic steps, and the follow-up data of the various treatment groups.

Group I (primary percutaneous, ultrasound-guided placement of a drainage): Nine patients of 27 (33.3%) underwent the approach of an external drainage of the PPC (Figure 1). In three patients, no further complaints were observed with no additional therapy. An internal drainage was placed in four patients (Figures 2 and 3). After the septic symptoms disappeared, the external drainage was replaced by an internal drainage (Figure 4). In the complication profile, there was one case of post-interventional bleeding with a subsequent 3-d stay at the ICU and discharge after a total of 40 d. In one case, septic shock occurred. The patient died after several open laparotomies for programmed lavages and necrectomy after a 74-d care at the ICU.

Group II (primary EUS-guided cystogastrostomy): By the mean of this approach, 13 of 27 patients (48.1%) were treated, aiming for the placement of an internal drainage for the PPC. In the vast majority of patients (n = 11),

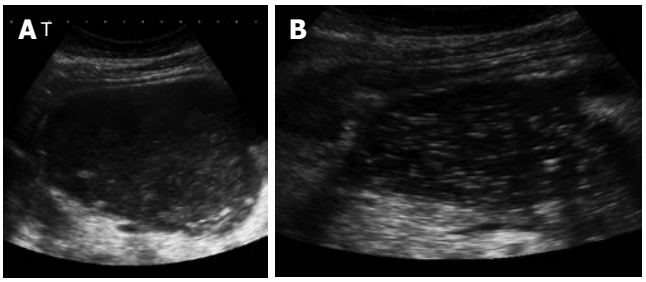

Figure 1 EUS images of an infected pancreatic pseudocyst (abscess) prior (**A**) and after placement (**B**) of an external drainage.

no further complaints or signs were reported, and no intervention-related symptoms were observed. Therefore, no further therapy was required. In two patients, an additional external drainage was placed: In one of the two patients, additional PPC were found, which, though communicating with other PPC, were not drained by the internally (transgastrocystically) placed drain reaching the originally detected PPC. In the second patient, a subsequent infection of the residual PPC was diagnosed following the initially successful internal drainage. This required a temporary external drainage. Out of 13 patients, there was only one serious complication. During the endoscopic intervention, a spontaneous perforation of the PPC was observed. Therefore, the cystogastrostomy was closed endoscopically by the mean of clips, which resulted in no further complaints, signs and symptoms after a 2-d stay and monitoring at the ICU. Hereafter, the patient´s

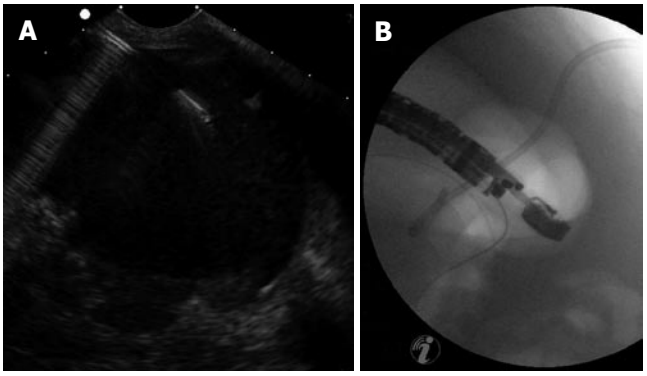

Figure 2 EUS-guided drainage of a pancreatic pseudocyst after previous external drainage: (**A**) EUS image; (**B**) Subsequent fluoroscopy control image of correct placement of the drainage.

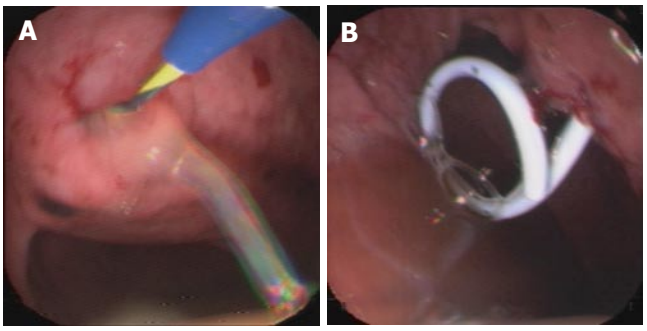

Figure 3 Endoscopy-guided cystogastrostomy via guide wire (**A**) and placement of an internal drainage (**B**).

discharge became possible.

Group III (EUS-guided cystogastrostomy including necrosectomy): This approach was used successfully in 5 of 27 patients (18.5 %) (Figure 5). The postinterventional course of all patients was uneventful and they did not show any problems or complications. On average, two endoscopic procedures (range, 2-6) were required to achieve the intended aim.

All together, there were three serious complications out of 27 subjects who underwent an interventional endoscopic approach of their PPC resulting in a complication rate of 11.1%. However, one patient died post-interventionally (mortality, 3.7%) because of septic complications with the underlying necrotizing pancreatitis.

DISCUSSION

In this report, treatment results for a novel interventional endoscopic approach for PPC are described. According to the aim of the study, this approach is a reasonable initial option in the therapeutic spectrum and it can be considered an establishing, less invasive, feasible, safe, and effective treatment alternative in experienced hands compared with open surgery, showing acceptable outcome and follow-up results with regard to complication rate and mortality[1-4,9-12]. The great advantage of this endoscopic approach is that it avoids the more severe trauma and invasiveness of a surgical intervention (no general

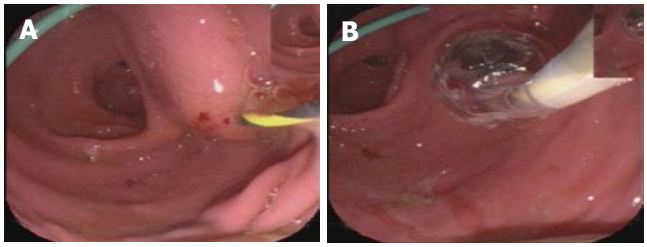

Figure 4 Endoscopic images: (**A**) EUS-guided puncture after previous external drainage; (**B**) Balloon dilatation via guide wire.

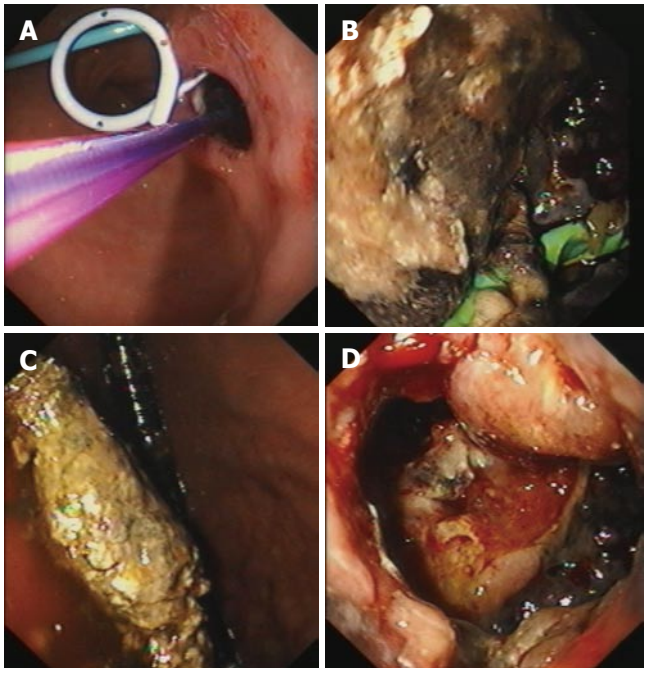

Figure 5 Endoscopic view: (**A**) After EUS-guided placement of an internal drainage (external drainage previously); (**B**) Into the pseudocystic cavity showing necroses; (**C**) Showing necrosectomy; (**D**) Through the transgastrocystic opening into the necrosectomized pseudocystic cavity.

anesthesia, no laparotomy, no risk for wound infection, less alteration of pulmonary and cardiac function, benefit for high-risk patients with a number of accompanying diseases or significant problems in their medical history). This endoscopic approach keeps the surgical intervention option still available in the case of unsuccessful outcome and follow-up. The endoscopic approach may provide comparable complication and mortality rates as shown for open cystogastrostomy or cystojejunostomy in a reasonably selected group of patients. However, the endoscopic approach has not changed but emphasizes the indications and principles[6] for the interventional treatment of PPC as follows: (1) Only symptomatic PPC are to be treated[6,12], (2) Basically, decision-making favors a forced interventional endoscopic approach together with an abdominal surgeon for surgical back-up[6]. (3) EUS is the main tool for diagnostic and treatment of PPC[3,4,6,10,13,14].

In particular, the novel aspect of the suggested approach is the less invasive character of this type of endoscopic intervention. The endoscopic approach

includes differential treatment options (primary percutaneous, ultrasound-guided placement of a drainage EUS-guided cystogastrostomy with or without subsequent necrosectomy) and a comparable outcome to open surgery as indicated by periinterventional morbidity and mortality.

The main complications and problems of the endoscopic procedure are subsequent infection of the cyst[6-9], bleeding[3,6-10], pancreatitis[7,8], perforation[9], colonic fistula[15], failure of the drainage[2], conversion[1], PPC recurrence[2,9,10,12], and even death. In our study, we observed only one bleeding, one perforation and one case of septic shock but none of the other possible complications. We showed a complication rate of 11.1% (n = 3), which is relatively low compared to De Palma *et al*[7,8] who reported a complication rate of 24.5%. In our study, there was one death due to necrotizing pancreatitis possibly indicating an unsuitable case for the endoscopic procedure.

According to this study, we suggest the following algorithm (a modification of Vosoghi[4] and Baillie[6]): (1) Initially, a possible malignant tumor growth of the cystic lesion needs to be excluded by means of EUS[6], laboratory analysis of cystic contents and cytologic investigation. In the case of an echogenic, thickened cystic wall and detectable Doppler signals within the cystic septum[6], a malignant cystic tumor lesion can be suspected. The suspected diagnosis can be supported by cytologic investigation of cystic content detecting tumor cells. In addition, levels of tumor markers such as CEA and Ca19-9 can be analyzed in the cystic fluid. The sensitivity and specificity of Ca19-9 to predict malignant tumor growth in cystic lesions of the pancreas is approximately 80% (cut off point, 50 000 IU/mL). In the case of a malignant cystic tumor lesion, a surgical approach is obligatory. If there are no hints of malignancy, (2) ERCP follows to clarify whether the PPC communicates with the pancreatic duct[6]. If yes, (3a) a transpapillary drainage needs to be implanted[4,6-10]. Otherwise, (3b) a transgastrocystic, EUS-guided drainage is placed[4,6-10]. If there are hints of an infected PPC or an abscess (echo-rich cystic content, necroses, sequester) revealed by transabdominal ultrasound, EUS, or clinical exam, primarily, (3c) an external (percutaneous) drainage of the PPC is to be favored[2,4]. After placement of a drainage, rinsing the cyst over several days and administration of antibiotics can be initiated. Depending on the patient´s clinical course (persisting PPC of the same size and configuration, persisting septic signs and symptoms, no falling inflammatory laboratory parameters) it has to be decided whether additionally, (3d) a necrosectomy[6] or even (3e) surgical intervention is required[4].

An interesting additional approach to consider is a laparoscopic endogastric pseudocyst gastrostomy, which combines less invasiveness, in particular, in large retrogastric PPC with the potential option of facilitated debridement of necrotic pancreas[1,2].

In conclusion, interventional endoscopic management of pancreatic pseudocysts is a reasonable alternative treatment option considering: (1) surgical principles[6] and (2) lower invasiveness.

Compared with surgical intervention there is a similar or even lower complication rate and mortality as shown by these study results. Subsequent follow-up investigations of the patients treated in this report, favoring again EUS, and studies with a higher value of evidence and larger case numbers are necessary to investigate treatment outcome and possible long-term consequences more appropriately[2,11].

REFERENCES

1 **Ammori BJ**, Bhattacharya D, Senapati PS. Laparoscopic endogastric pseudocyst gastrostomy: a report of three cases. *Surg Laparosc Endosc Percutan Tech* 2002; **12**: 437-440

2 **Bhattacharya D**, Ammori BJ. Minimally invasive approaches to the management of pancreatic pseudocysts: review of the literature. *Surg Laparosc Endosc Percutan Tech* 2003; **13**: 141-148

3 **Giovannini M**, Binmoeller K, Seifert H. Endoscopic ultrasound-guided cystogastrostomy. *Endoscopy* 2003; **35**: 239-245

4 **Vosoghi M**, Sial S, Garrett B, Feng J, Lee T, Stabile BE, Eysselein VE. EUS-guided pancreatic pseudocyst drainage: review and experience at Harbor-UCLA Medical Center. *MedGenMed* 2002; **4**: 2

5 **Baillie J**. Pancreatic pseudocysts (Part I). *Gastrointest Endosc* 2004; **59**: 873-879

6 **Baillie J**. Pancreatic pseudocysts (Part II). *Gastrointest Endosc* 2004; **60**: 105-113

7 **De Palma GD**, Galloro G, Puzziello A, Masone S, Diamantis G, Persico G. [Personal experience with the endoscopic treatment of pancreatic pseudocysts. Long-term results and analysis of prognostic factors]. *Minerva Chir* 2001; **56**: 475-481

8 **De Palma GD**, Galloro G, Puzziello A, Masone S, Persico G. Endoscopic drainage of pancreatic pseudocysts: a long-term follow-up study of 49 patients. *Hepatogastroenterology* 2002; **49**: 1113-1115

9 **Sharma SS**, Bhargawa N, Govil A. Endoscopic management of pancreatic pseudocyst: a long-term follow-up. *Endoscopy* 2002; **34**: 203-207

10 **Dohmoto M**, Akiyama K, Lioka Y. Endoscopic and endosonographic management of pancreatic pseudocyst: a long-term follow-up. *Rev Gastroenterol Peru* 2003; **23**: 269-275

11 **Seifert H**, Wehrmann T, Schmitt T, Zeuzem S, Caspary WF. Retroperitoneal endoscopic debridement for infected peripancreatic necrosis. *Lancet* 2000; **356**: 653-655

12 **Yang CC**, Shin JS, Liu YT, Yueh SK, Chou DA. Management of pancreatic pseudocysts by endoscopic cystogastrostomy. *J Formos Med Assoc* 1999; **98**: 283-286

13 **Norton ID**, Clain JE, Wiersema MJ, DiMagno EP, Petersen BT, Gostout CJ. Utility of endoscopic ultrasonography in endoscopic drainage of pancreatic pseudocysts in selected patients. *Mayo Clin Proc* 2001; **76**: 794-798

14 **Seifert H**, Faust D, Schmitt T, Dietrich C, Caspary W, Wehrmann T. Transmural drainage of cystic peripancreatic lesions with a new large-channel echo endoscope. *Endoscopy* 2001; **33**: 1022-1026

15 **Howell DA**, Dy RM, Gerstein WH, Hanson BL, Biber BP. Infected pancreatic pseudocysts with colonic fistula formation successfully managed by endoscopic drainage alone: report of two cases. *Am J Gastroenterol* 2000; **95**: 1821-1823

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RAPID COMMUNICATION

Prevalence of factor V Leiden and prothrombin G20210A in patients with gastric cancer

Sandra Battistelli, Massimo Stefanoni, Alberto Genovese, Aurelio Vittoria, Roberto Cappelli, Franco Roviello

Sandra Battistelli, Massimo Stefanoni, Alberto Genovese, Dipartimento di Chirurgia e Specialità Chirurgiche, Policlinico Le Scotte, Viale Bracci, 53100 Siena, Italy
Aurelio Vittoria, Dipartimento di Medicina Clinica e Scienze Immunologiche, Policlinico Le Scotte, Viale Bracci, 53100 Siena, Italy
Roberto Cappelli, Dipartimento di Medicina Interna, Cardiovascolare e Geriatrica, Policlinico Le Scotte, Viale Bracci, 53100 Siena, Italy
Franco Roviello, Dipartimento di Patologia Umana ed Oncologia, Policlinico Le Scotte, Viale Bracci, 53100 Siena, Italy
Supported by a Research Grant from the University of Siena (PAR)
Correspondence to: Franco Roviello, MD, Via A. De Gasperi 5, 53100 Siena, Italy. roviello@unisi.it
Telephone: +39-577-585157 Fax: +39-577-585157
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Abstract

AIM: To analyze the prevalence of the two commonest thrombophilic mutations, factor V Leiden and prothrombin G20210A, in patients with gastric cancer.

METHODS: One hundred and twenty-one patients with primary gastric carcinoma and 130 healthy subjects, comparable for age and sex, were investigated. Factor V Leiden was detected by using polymerase chain reaction and restriction enzyme digestion, and prothrombin G20210A gene mutation by allele-specific PCR.

RESULTS: Among the 121 cancer patients, factor V Leiden was found in 4 cases (GA genotype: 3.3%) and prothrombin G20210A in 10 cases (GA genotype: 8.3%). Of the 130 control subjects, factor V Leiden was detected in 6 cases (GA genotype: 4.6%) and prothrombin G20210A in 8 cases (GA genotype: 6.1%). No double heterozygous carriers of both mutations were found in either group. The prevalence of both factor V Leiden and prothrombin G20210A variant was not statistically different between the cancer patients and the healthy subjects.

CONCLUSION: Our study suggests that, in gastric cancer, the risk factors of thrombophilic cancer state are on acquired rather than on a genetic basis and that prothrombin G20210A does not seem to be a cofactor in gastric cancer pathogenesis.

Key words: Gastric cancer; Genetic polymorphism; Factor V Leiden; Prothrombin G20210A

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INTRODUCTION

The pathogenesis of haemostatic disorders in cancer is complex and mainly on acquired basis. To date, little conclusive information is available in literature about the association of cancer hypercoagulability and inherited thrombophilia. The most common genetic defect in Caucasian population is factor V Leiden, a glutamine to arginine switch at amino-acid 506, due to G to A transition at nucleotide 1691 of coagulation factor V. This point mutation makes factor Va resistant to the proteolytic action of activated protein C so that activated factor V persists, rather than being inactivated. Another genetic defect is prothrombin G20210A polymorphism, a G to A transition at nucleotide 20210 in the 3'-untranslated region of the prothrombin gene, associated with elevated levels of prothrombin, which contributes to thrombotic risk by promoting enhanced thrombin generation.

Recently, Miller *et al*[1] found an increased incidence of neoplasia of the digestive tract in men with persistent activation of the coagulation pathway. In addition, there is some evidence that prothrombin G20210A gene mutation may be involved in cancer development and/or progression. The prevalence of factor V Leiden and prothrombin G20210A gene polymorphism was analyzed in a cohort of 175 patients with gastrointestinal carcinoma and a significantly increased prevalence of prothrombin gene mutation in the patient group as compared to normal controls was detected[2]). On the contrary, Paspatis *et al*[3] demonstrated that the prevalence of both factor V Leiden and prothrombin G20210A in 74 colorectal cancer patients was found to be similar to that of 192 colonoscopically selected control subjects. To the best of our knowledge, no data regarding the prevalence of factor V Leiden and prothrombin G20210A in the subset of patients with gastric cancer are available in the literature. We, therefore, performed a prospective case-control study to analyze the

prevalence of factor V Leiden and prothrombin G20210A in patients with gastric cancer.

MATERIALS AND METHODS

One hundred and twenty-one consecutive patients (78 men, 43 women; mean age: 62 years, range: 51-76 years) with operable gastric carcinoma (TNM staging: T_{1-3}, N_{0-2}, M_0) and 130 healthy subjects, matched for age, sex and ethnic-background, were investigated for the presence of factor V Leiden and prothrombin G20210A gene mutation. All individuals were from central Italy, without any previous thrombotic event. Specifically, no control subject or cancer patient had a history of peptic ulcer or *H pylori* infection.

Genomic DNA was extracted from white blood cells according to standard procedures. Factor V Leiden was detected by using polymerase chain reaction and restriction enzyme digestion following the methods described by Bertina *et al*[4]. Prothrombin G20210A gene mutation was detected by allele-specific PCR according to the methods described by Poort *et al*[5].

Statistical analysis

Statistical analysis was performed using χ^2 test (with Yates correction).

RESULTS

Among the 121 cancer patients, factor V Leiden was found in 4 cases (GA genotype: 3.3%) and prothrombin G20210A variant in 10 cases (GA genotype: 8.3%). Among the 130 control subjects, factor V Leiden was detected in 6 cases (GA genotype: 4.6%) and prothrombin G20210A variant in 8 cases (GA genotype: 6.1%). No double heterozygous carriers of both mutations were found in both groups. The prevalence of both factor V Leiden and prothrombin G20210A was not statistically different between the cancer patients and the healthy subjects.

DISCUSSION

The present study suggests that, in gastric cancer patients, there is no increase in the prevalence of both factor V Leiden and prothrombin G20210A gene mutation. These data seem apparently in contrast with the results of Pihusch *et al*[1], obtained in a cohort of patients with carcinoma of all the gastrointestinal tract (most patients with adenocarcinoma of the colon), and this could indicate a different, peculiar pathogenetic pathway of gastric carcinogenesis. To our knowledge, this is the first report about the prevalence of factor V Leiden and prothrombin G20210A in a cohort of gastric cancer patients. Moreover, the ethnic background of our subjects is different and this could partly explain our results. On the other hand, our data are in agreement with the findings by Paspatis *et al*[2] who did not find a significant difference in the prevalence of the two prothrombotic polymorphisms in 74 colorectal cancer patients as compared to the controls. However, the limitation of our study is the low statistical power of our sample size.

In conclusion, our data strengthed the evidence that, in gastric cancer, the thrombophilic state is on acquired rather than on genetic basis and seem to suggest that the prothrombin G20210A is not involved in gastric cancer pathogenesis.

REFERENCES

1 **Miller GJ**, Bauer KA, Howarth DJ, Cooper JA, Humphries SE, Rosenberg RD. Increased incidence of neoplasia of the digestive tract in men with persistent activation of the coagulant pathway. *J Thromb Haemost* 2004; **2**: 2107-2114

2 **Pihusch R**, Danzl G, Scholz M, Harich D, Pihusch M, Lohse P, Hiller E. Impact of thrombophilic gene mutations on thrombosis risk in patients with gastrointestinal carcinoma. *Cancer* 2002; **94**: 3120-3126

3 **Paspatis GA**, Sfyridaki A, Papanikolaou N, Triantafyllou K, Livadiotaki A, Kapsoritakis A, Lydataki N. Resistance to activated protein C, factor V leiden and the prothrombin G20210A variant in patients with colorectal cancer. *Pathophysiol Haemost Thromb* 2002; **32**: 2-7

4 **Bertina RM**, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-67

5 **Poort SR**, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; **88**: 3698-3703

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Mass-forming pancreatitis: Value of contrast-enhanced ultrasonography

Mirko D'Onofrio, Giulia Zamboni, Alessia Tognolini, Roberto Malagò, Niccolò Faccioli, Luca Frulloni, Roberto Pozzi Mucelli

Mirko D'Onofrio, Giulia Zamboni, Alessia Tognolini, Roberto Malago', Niccolo' Faccioli, Roberto Pozzi Mucelli, Department of Radiology, University of Verona, Verona, Italy
Luca Frulloni, Department of Gastroenterology, University of Verona, Verona, Italy
Correspondence to: Mirko D'Onofrio, MD, Department of Radiology, University of Verona, Policlinico G.B. Rossi, Piazzale L.A. Scuro 37134 Verona, Italy. mirko.donofrio@univr.it
Telephone: +39-45-8074301 Fax: +39-45-8277808
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Abstract

AIM: To assess the utility of contrast-enhanced ultrasonography (CEUS) with a second-generation contrast medium in the differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma.

METHODS: From our radio-pathology database, we retrieved all the patients affected by mass-forming pancreatitis or pancreatic carcinoma who underwent CEUS. We evaluated the results of CEUS in the study of the 173 pancreatic masses considering the possibilities of a differential diagnosis between mass-forming pancreatitis and pancreatic tumor by identifying the "parenchymographic" enhancement during the dynamic phase of CEUS, which was considered diagnostic for mass-forming pancreatitis.

RESULTS: At CEUS, 94% of the mass-forming pancreatitis showed intralesional parenchymography. CEUS allowed diagnosis of mass-forming pancreatitis with sensitivity of 88.6%, specificity of 97.8%, positive predictive value of 91.2%, negative predictive value of 97.1%, and overall accuracy of 96%. CEUS significantly increased the diagnostic confidence in the differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma, with receiver operating characteristic curve areas from 0.557 (P = 0.1608) for baseline US to 0.956 (P < 0.0001) for CEUS.

CONCLUSION: CEUS allowed diagnosis of mass-forming pancreatitis with diagnostic accuracy of 96%. CEUS significantly increases the diagnostic confidence with respect to basal US in discerning mass-forming pancreatitis from pancreatic neoplasm.

Key words: Contrast-enhanced ultrasonography; Pancreatitis; Pancreatic neoplasm; Mass-forming pancreatitis

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INTRODUCTION

Mass-forming pancreatitis usually arises in patients with a history of chronic pancreatitis[1]. The main characteristic feature at pathology is progressive interstitial fibrosis with chronic inflammatory infiltrate. Differential diagnosis with a neoplastic disease may be difficult because mass-forming pancreatitis and pancreatic tumor may present with the same symptoms and signs[2]. Autoimmune pancreatitis is a rare type of chronic pancreatitis which has been proposed as a separate clinical entity in 1995 and later defined[3,4]. As opposed to the other forms of chronic pancreatitis, in the autoimmune form, the pancreas is increased in volume, usually in a diffuse way with the typical "sausage" look, and Wirsung duct is compressed by glandular parenchyma or string-like[4]. At ultrasonography (US), mass-forming pancreatitis is often very similar to pancreatic carcinoma[1,5], presenting in most cases as a hypoechoic mass in a limited sector of the gland, usually at the head, often with enlargement or lumpiness of the gland contour. Contrast-enhanced ultrasonography (CEUS), thanks to the real-time continuous visualization of blood perfusion of the pancreas and its masses, has been recently used in the evaluation of the vascularization of solid pancreatic lesions, with results superior to single-slice spiral CT[6,7]. The CEUS features of autoimmune pancreatitis have also been evaluated[8]. In this study, we aimed to assess the utility of CEUS with a second-generation contrast medium in the differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma.

MATERIALS AND METHODS

From our radio-pathology database, we retrieved all the patients affected by mass-forming pancreatitis or pancreatic ductal adenocarcinoma who underwent CEUS at our Institution between January 2002 and January 2005. Our Institutional Review Board does not require any informed consent for retrospective studies. This study included 35

patients (26 males, 9 females, mean age 49.1 years) affected by mass-forming pancreatitis (19 chronic alcohol-related pancreatitis, 15 chronic autoimmune pancreatitis, 1 genetic pancreatitis) and 138 patients affected by pancreatic tumors (78 males, 60 females, mean age 62.4 years). A total of 173 pancreatic masses were enrolled. All the pancreatic masses underwent cytological or histological diagnosis. All the patients with cytological diagnosis of pancreatitis were followed up at least for one year. All CEUS examinations were performed by radiologists on a Sequoia 512 6.0 (Acuson, Mountain View, CA, USA) ultrasound system, with harmonic microbubble-specific imaging with low acoustic ultrasound pressure (2-4 MHz Coherent Contrast Imaging or Cadence Contrast Pulse Sequencing; Mechanical Index 0.2; 12-13 frames/s). A 2.4 mL bolus of a second-generation contrast medium, SonoVue® (Bracco, Milan, Italy), was intravenously injected, followed by a 5 mL bolus of saline solution. All CEUS examinations were performed by the same radiologist and recorded on videotape/VHS or magneto optical disk/MOD systems to have the possibility to immediately review the dynamic study. Insonation of the pancreatic lesion was continuous with dynamic observation of the shift from the unenhanced phase to the contrast-enhanced phase. The enhancement pattern of the lesions was compared to that of the adjacent normal parenchyma. Definition of the arterial phase is possible when observing hyperechogenity of the aorta or other big perilesional arteries. The venous phase is defined when the splenomesenteric-portal tree becomes hyperechoic. The lesions were classified according to the lesional enhancement in the enhanced phases as hypovascular/hypoechoic (lesions almost without enhancement or with enhancement lower than that of the adjacent parenchyma), isovascular/isoechoic (lesions with slight continuous enhancement or enhancement similar to that of the adjacent parenchyma) and hypervascular/hyperechoic (lesions with bright enhancement or enhancement superior to that of the adjacent parenchyma). The presence of a slight continuous enhancement inside the pancreatic masses, isovascular with the adjacent pancreatic parenchyma, was defined as "parenchymographic enhancement". We evaluated the results of CEUS in the study of the 173 pancreatic masses considering the possibilities of a differential diagnosis between mass-forming pancreatitis and pancreatic tumor by identifying the parenchymographic enhancement during the dynamic phase of CEUS, which was considered diagnostic for mass-forming pancreatitis. The reports of all the CEUS examinations were reviewed retrospectively, but all the CEUS studies had been interpreted in a prospective manner by the attending radiologist and so utilized for the data analysis. The sensitivity, specificity, positive and negative predictive values and diagnostic accuracy of CEUS in the characterization of the pancreatic masses were then calculated. Moreover, to compare baseline US and CEUS, each mass-forming pancreatitis was evaluated at baseline US and at CEUS with a 3 level diagnostic score: 0 = absence of malignancy (isoechoic lesions); 1 = indeterminate (hyperechoic lesions); and 2 = presence of malignancy (hypoechoic lesions). Diagnostic confidences of baseline US and of CEUS in the characterization of mass-forming pancreatitis were represented by means of the ROC (receiver operating characteristic) curves; diagnostic advantage of CEUS on baseline US was calculated by comparing the areas under the ROC curves obtained by using a computer software package (Analise-it; Analise-it-software, Leeds, England).

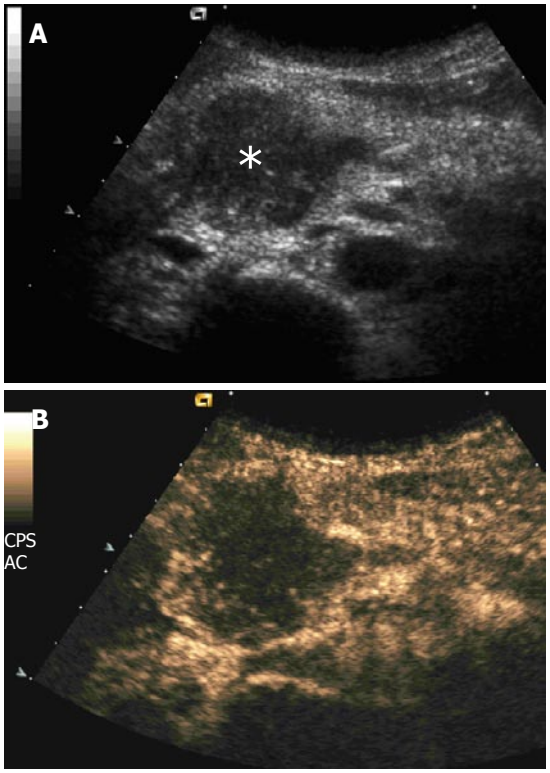

Figure 1 Ductal adenocarcinoma. **A**: US showing slightly hypoechoic pancreatic head mass (asterisk); **B**: CEUS showing poor enhancement of the mass, appearing hypoechoic to the rest of pancreatic parenchyma in the contrast-enhanced phases.

RESULTS

All the contrast-enhanced ultrasound examinations were technically adequate allowing the dynamic observation of the shift from the unenhanced to the contrast-enhanced arterial and venous phases.

Pancreatic tumors

Pancreatic carcinomas were hypoechoic to the adjacent parenchyma at CEUS during the dynamic phases in 91% (126/138) of the cases (Figure 1), while hyperechoic and isoechoic in 7% (9/138) and 2% (3/138) of the cases, respectively.

Mass-forming pancreatitis

Mass-forming pancreatitis involved diffusely the pancreatic parenchyma in 8.5% (10/35) of the cases, while 71.5% (25/35) of the cases were focally localized. Of the 25 focally localized cases, 16 were at the pancreatic head, 5 at the uncinate process and 4 at the pancreatic body. The main pancreatic duct was dilated in 10 cases, the common bile duct was dilated in 11, and dilation of both ducts was observed in 6 cases. Lesional calcifications were

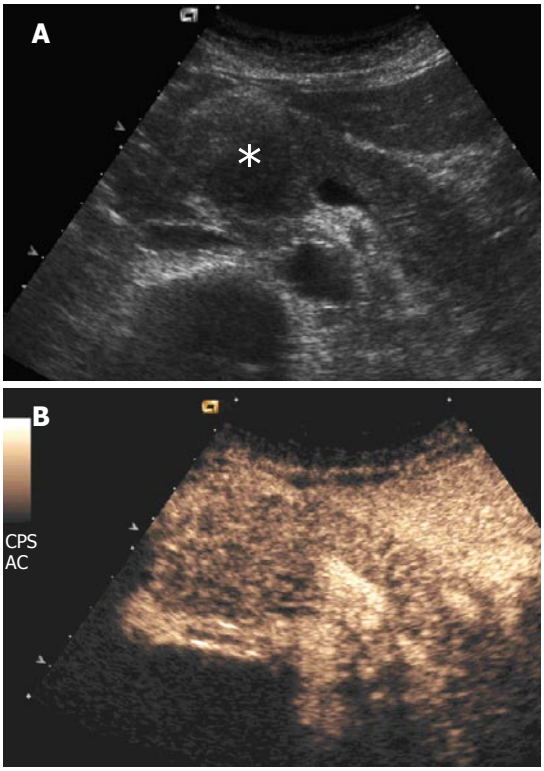

Figure 2 Mass-forming chronic autoimmune pancreatitis. **A**: US showing hypoechoic head pancreatic mass (asterisk); **B**: CEUS showing parenchymographic enhancement of the pancreatic lesion in the head of the pancreas during the contrast-enhanced phases.

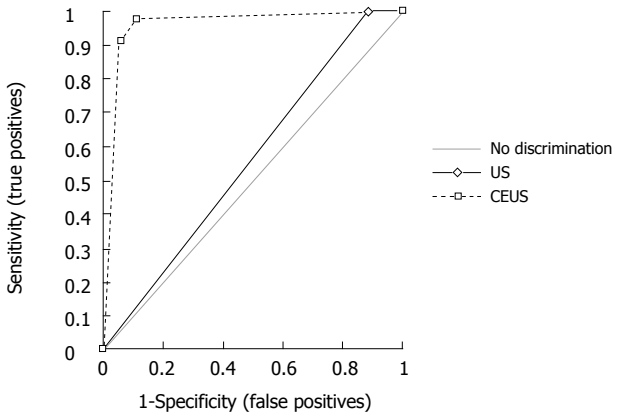

Figure 3 Receiver operating characteristic curves of baseline ultrasound and contrast-enhanced ultrasound in the characterization of 173 pancreatic masses, discerning between benignancy (pancreatitis) and malignancy (tumor).

observed in 5 (14%) patients. One patient was positive for the SPINK-1 genetic marker. Five patients had significantly higher levels of serum tumoral markers (CA 125 in 1 and CA 19-9 in 4 cases). At CEUS, 94% of the mass-forming pancreatitis showed intralesional glandular parenchymography (Figure 2), while 6% of the mass-forming pancreatitis remained hypoechoic during the dynamic phase. CEUS allowed diagnosis of mass-forming pancreatitis, assuming isoechogenicity as significant for pancreatitis, with sensitivity of 88.6%, specificity of 97.8%, positive predictive value of 91.2%, negative predictive value of 97.1%, and overall accuracy of 96%. The presence of parenchymographic enhancement or hypoechogenicity in the examined pancreatic masses in the dynamic phases of CEUS significantly increased the diagnostic confidence in the differential diagnosis between mass-forming pancreatitis and pancreatic tumor, with receiver operating characteristic curve areas from 0.557 (P = 0.1608) for baseline US to 0.956 ($P < 0.0001$) for CEUS (Figure 3).

DISCUSSION

Mass-forming pancreatitis is caused by various etiopathogenetic factors. However, at least two distinct categories have been recognized: alcohol-induced and autoimmune-related[9]. Differential diagnosis between mass-forming pancreatitis and pancreatic tumor is a crucial point for the correct management of patients affected by pancreatic masses. However, differential diagnosis can be difficult in clinical practice[10,11]. In fact, mass-forming pancreatitis and pancreatic tumors may present with the same symptoms and signs[2]. Several clinical and imaging features have been proposed to be helpful for the differential diagnosis. In case of mass-forming chronic pancreatitis, the presence of small calcifications in the lesion may suggest its inflammatory nature[12], but is surely poorly specific. The identification of perfusion features similar to those of the normal pancreatic parenchyma (i.e., glandular parenchymography) is strongly suggestive of inflammatory mass, while ductal carcinomas typically display a low/absent enhancement due to the scirrhous content of this tumor. Contrast-enhanced ultrasonography (CEUS) has recently been used for pancreatic lesions study with good results[6,13]. The CEUS finding consistent with an inflammatory origin of a pancreatic mass is the presence of a parenchymographic enhancement, defined as slight continuous enhancement inside the pancreatic mass with isovascularity to the adjacent pancreatic parenchyma. In this study, considering the presence of this sign, CEUS diagnosis of mass-forming pancreatitis was possible with sensitivity of 88.6%, specificity of 97.8%, positive predictive value of 91.2%, negative predictive value of 97.1% and an overall diagnostic accuracy of 96%. The intensity of this parenchymographic enhancement is surely related to the length of the underlying inflammatory process. It has been observed that the more the inflammatory process is chronic and long-standing, the less intense is the intralesional parenchymography, probably in relation to the entity of the associated fibrosis. As opposed to this, in mass-forming pancreatitis of more recent onset, the enhancement is usually more intense and prolonged[5]. The characteristic findings of autoimmune pancreatitis have been well defined: increased levels of serum gammaglobulin or IgG; presence of autoantibodies; enlargement of the pancreas; diffusely irregular narrowing of the main pancreatic duct and occasional stenosis of the intrapancreatic bile duct; fibrotic changes with lymphocyte infiltration; absent or mild symptoms; rare pancreatic calcifications and cysts; occasional association with other autoimmune diseases and effective steroid therapy[14]. Autoimmune pancreatitis is also reported to be characterized

by periductal flogosis, mainly sustained by lymphocytic infiltration, with evolution to fibrosis[3,4]. The exact incidence and prevalence of this disease are not known, but previous studies have shown a male preponderance (ratio of 2:1) and a predominant involvement of the elderly age group[15]. The association with other autoimmune diseases has been reported, although data about the exact incidence of this association are not available[14]; the most commonly associated diseases are Sjogren syndrome, diabetes mellitus, inflammatory bowel disease (especially Crohn's disease), primary biliary cirrhosis, primary sclerosing cholangitis and systemic lupus erythematosus[15,16]. Response to steroid therapy is reported in the literature, but dosage and duration of the therapy are not standardized. However, pancreatitis is the most common benign condition that mimics pancreatic neoplasm, and in recent experience at Johns Hopkins, lymphoplasmacytic sclerosing pancreatitis is the most common form of pancreatitis in patients who are subjected to pancreaticduodenectomy for suspected neoplasm[17]. Ultrasonographic features of autoimmune pancreatitis are very similar to those of focal pancreatitis, even though autoimmune pancreatitis may interest more frequently the entire gland or present a larger extension and ubiquitous localization. US findings are characteristic in the diffuse form when the entire gland is involved. Echogenicity is markedly reduced, gland volume is increased and Wirsung duct is compressed by glandular parenchyma. Focal autoimmune pancreatitis at the pancreatic head is often characterized by the sole dilation of the common bile duct[8]. The vascularization of autoimmune pancreatitis can be demonstrated by CEUS, which shows most often a moderate or marked[8] enhancement in the early contrast-enhanced phase, though inhomogeneous due to the thinning of the glandular vessels as the consequence of the thick lymphocytic infiltration and fibrosis. In our study, parenchymographic enhancement, defined as slight continuous enhancement inside the pancreatic masses with isovascularity to the adjacent pancreatic parenchyma, was shown in 94% of the mass-forming pancreatitis. These CEUS findings have been reported to be especially useful in the study of focal forms of autoimmune chronic pancreatitis, in which differential diagnosis with ductal carcinoma is a priority[11]. The ability of EUS as well other imaging modalities to differentiate pancreatic cancer from pseudotumorous chronic pancreatitis is reported in literature[18]. Our data suggests that contrast-enhanced ultrasonography should be used in the characterization of pancreatic masses as complementary to CT and MRI. The sensitivity of CEUS in the identification of inflammatory masses allows to propose to obtain a diagnosis with fine needle percutaneous cytology in pancreatic focal masses that show glandular parenchymography at the examination. However, our study is surely limited by the retrospective evaluation.

In conclusion, CEUS allows diagnosis of mass-forming pancreatitis with diagnostic accuracy of 96%. CEUS significantly increases the diagnostic confidence in respect to basal US in discerning mass-forming pancreatitis from pancreatic neoplasm.

REFERENCES

1 **Kim T**, Murakami T, Takamura M, Hori M, Takahashi S, Nakamori S, Sakon M, Tanji Y, Wakasa K, Nakamura H. Pancreatic mass due to chronic pancreatitis: correlation of CT and MR imaging features with pathologic findings. *AJR Am Roentgenol* 2001; **177**: 367-371

2 **van Gulik TM**, Reeders JW, Bosma A, Moojen TM, Smits NJ, Allema JH, Rauws EA, Offerhaus GJ, Obertop H, Gouma DJ. Incidence and clinical findings of benign, inflammatory disease in patients resected for presumed pancreatic head cancer. *Gastrointest Endosc* 1997; **46**: 417-423

3 **Yoshida K**, Toki F, Takeuchi T, Watanabe S, Shiratori K, Hayashi N. Chronic pancreatitis caused by an autoimmune abnormality. Proposal of the concept of autoimmune pancreatitis. *Dig Dis Sci* 1995; **40**: 1561-1568

4 **Furukawa N**, Muranaka T, Yasumori K, Matsubayashi R, Hayashida K, Arita Y. Autoimmune pancreatitis: radiologic findings in three histologically proven cases. *J Comput Assist Tomogr* 1998; **22**: 880-883

5 **Koito K**, Namieno T, Nagakawa T, Morita K. Inflammatory pancreatic masses: differentiation from ductal carcinomas with contrast-enhanced sonography using carbon dioxide microbubbles. *AJR Am J Roentgenol* 1997; **169**: 1263-1267

6 **D'Onofrio M**, Malagò R, Zamboni G, Vasori S, Falconi M, Capelli P, Mansueto G. Contrast-enhanced ultrasonography better identifies pancreatic tumor vascularization than helical CT. *Pancreatology* 2005; **5**: 398-402

7 **Kitano M**, Kudo M, Maekawa K, Suetomi Y, Sakamoto H, Fukuta N, Nakaoka R, Kawasaki T. Dynamic imaging of pancreatic diseases by contrast enhanced coded phase inversion harmonic ultrasonography. *Gut* 2004; **53**: 854-859

8 **Numata K**, Ozawa Y, Kobayashi N, Kubota T, Akinori N, Nakatani Y, Sugimori K, Imada T, Tanaka K. Contrast-enhanced sonography of autoimmune pancreatitis: comparison with pathologic findings. *J Ultrasound Med* 2004; **23**: 199-206

9 **Wakabayashi T**, Kawaura Y, Satomura Y, Watanabe H, Motoo Y, Okai T, Sawabu N. Clinical and imaging features of autoimmune pancreatitis with focal pancreatic swelling or mass formation: comparison with so-called tumor-forming pancreatitis and pancreatic carcinoma. *Am J Gastroenterol* 2003; **98**: 2679-2687

10 **Kamisawa T**, Egawa N, Nakajima H. Autoimmune pancreatitis is a systemic autoimmune disease. *Am J Gastroenterol* 2003; **98**: 2811-2812

11 **Koga Y**, Yamaguchi K, Sugitani A, Chijiiwa K, Tanaka M. Autoimmune pancreatitis starting as a localized form. *J Gastroenterol* 2002; **37**: 133-137

12 **Remer EM**, Baker ME. Imaging of chronic pancreatitis. *Radiol Clin North Am* 2002; **40**: 1229-1242, v

13 **D'Onofrio M**, Mansueto G, Falconi M, Procacci C. Neuroendocrine pancreatic tumor: value of contrast enhanced ultrasonography. *Abdom Imaging* 2004; **29**: 246-258

14 **Okazaki K**, Chiba T. Autoimmune related pancreatitis. *Gut* 2002; **51**: 1-4

15 **Klöppel G**, Lüttges J, Löhr M, Zamboni G, Longnecker D. Autoimmune pancreatitis: pathological, clinical, and immunological features. *Pancreas* 2003; **27**: 14-19

16 **Okazaki K**. Autoimmune pancreatitis: etiology, pathogenesis, clinical findings and treatment. The Japanese experience. *JOP* 2005; **6**: 89-96

17 **Kawamoto S**, Siegelman SS, Hruban RH, Fishman EK. Lymphoplasmacytic sclerosing pancreatitis with obstructive jaundice: CT and pathology features. *AJR Am J Roentgenol* 2004; **183**:915-921

18 **Dancygier H**, Lightdale CJ. Endoscopic ultrasonography of the upper gastrointestinal tract and colon. In: Stevens PD, ed. Endosonography in Gastroenterology: principles, Techniques, Findings. New York: Thieme; 1999: 13-173

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wjg@wjgnet.com

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RAPID COMMUNICATION

Predictive factors of survival in patients treated with definitive chemoradiotherapy for squamous cell esophageal carcinoma

Frédéric Di Fiore, Stéphane Lecleire, Olivier Rigal, Marie-Pierre Galais, Emmanuel Ben Soussan, Isabelle David, Bernard Paillot, Jacques-Henri Jacob, Pierre Michel

Frédéric Di Fiore, Stéphane Lecleire, Olivier Rigal, Emmanuel Ben Soussan, Bernard Paillot, Pierre Michel, Digestive Oncology Unit, Hepatogastroenterology Department, Rouen University Hospital, France
Marie-Pierre Galais, Jacques-Henri Jacob, Digestive Oncology unit, François Baclesse Centre, Caen, France
Isabelle David, Radiotherapy Department, Henri Becquerel Centre, Rouen, France
Correspondence to: Dr. Frédéric Di Fiore, Digestive Oncology Unit, Hepatogastroenterology Department, Rouen University Hospital- Charles Nicolle- France, 1, rue de Germont, 6031 Rouen Cedex, France. eliottdf@hotmail.com
Telephone: +33-2-32888101 Fax: +33-2-35151623
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Di Fiore F, Lecleire S, Rigal O, Galais MP, Ben Soussan E, David I, Paillot B, Jacob JH, Michel P. Predictive factors of survival in patients treated with definitive chemoradiotherapy for squamous cell esophageal carcinoma. *World J Gastroenterol* 2006; 12(26): 4185-4190

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Abstract

AIM: The aim of the study was to evaluate the predictive factors of survival in patients with locally advanced squamous cell esophageal carcinoma (LASCOC) treated with definitive chemoradiotherapy (CRT) regimen based on the 5FU/CDDP combination.

METHODS: All patients with LASCOC treated with a definitive CRT using the 5FU/CDDP combination between 1994 and 2000 were retrospectively included. Clinical complete response (CCR) to CRT was assessed by esophageal endoscopy and CT-scan 2 mo after CRT completion. Prognostic factors of survival were assessed using univariate and multivariate analysis by the Cox regression model.

RESULTS: A total of 116 patients were included in the study. A CCR to CRT was observed in 86/116 (74.1%). The median survival was 20 mo (range 2-114) and the 5-year survival was 9.4%. Median survival of responder patients to CRT was 25 mo (range 3-114) as compared to 9 mo (range 2-81) in non-responder patients ($P < 0.001$). In univariate analysis, survival was associated with CCR ($P < 0.001$), WHO performance status < 2 ($P = 0.01$), tumour length < 6 cm ($P = 0.045$) and weight loss < 10% was in limit of significance ($P = 0.053$). In multivariate analysis, survival was dependant to CCR ($P < 0.0001$), weight loss < 10% ($P = 0.034$) and WHO performance < 2 ($P = 0.046$).

CONCLUSION: Our results suggest that survival in patients with LASCOC treated with definitive CRT was correlated to CCR, weight loss and WHO performance status.

INTRODUCTION

Esophageal cancer is a frequent gastrointestinal malignancy with 32 332 new cases per year in Europe. In France, esophageal carcinoma is the third most frequent digestive tract cancer with approximately 5000 new cases per year[1,2]. Approximately 50% of patients present a locally advanced esophageal carcinoma at diagnosis. To date, the incidence rate of adenocarcinoma is increasing but squamous cell carcinoma still remains the most frequent histological type in France[1,2]. The definitive chemoradiotherapy (CRT) based on the Herskovic regimen is considered as the standard medical treatment in non operated patients with locally advanced esophageal carcinoma[3,4]. Moreover, two phase III trials recently suggested that definitive CRT could be considered as an alternative treatment in patients with esophageal carcinoma[5,6].

However, some questions remain unsolved as regards the CRT regimen optimisation. Moreover, although most studies included both patients with squamous cell and adenocarcinoma, it has been suggested to consider these two tumours separately for treatment as regards their different risk factors, carcinogenesis pathways and treatment response[3-5,7-11]. Furthermore, the study of Rizk *et al* recently reported that long term prognosis in patients with esophageal carcinoma treated with preoperative CRT could be significantly different according to the histological type of tumour[11]. However, few reported series have specifically focused on the long-term survival analysis in patients with squamous cell carcinoma treated with definitive CRT using the 5FU/CDDP combination[12-20]. Moreover, most of these series included a limited number of patients or reported results with short follow-up[13-20].

The aim of the present study was to assess the long-

term results and the predictive factors of survival in a large series of patients with a locally advanced squamous cell esophageal carcinoma (LASCOC) treated with a definitive CRT regimen based on the 5FU/CDDP chemotherapy (CT) combination. Furthermore, knowledge of these prognostic factors could be useful for the management of individual patients as well as a stratification variable for the design of future randomised trials.

MATERIALS AND METHODS

Patient population

All consecutive patients with a LASCOC referred between January 1994 and the 31st December 2000 were retrospectively included for the study. Patients were selected based on the following criteria: a histologically confirmed squamous cell carcinoma; a first-line treatment with a definitive CRT regimen using the 5FU/CDDP CT and concomitant external radiotherapy (RT). Patients were excluded if they had previous a history of carcinoma during the past three years and if they had synchronous distant metastasis. In our centres, the definitive CRT regimen based on the 5FU/CDDP combination was the first therapeutic option used in patients with LASCOC.

For each patient, we routinely recorded all baseline clinical and tumour characteristics including age, sex, World Heath Organisation (WHO) performance status, dysphagia Atkinson score and weight loss at the beginning of treatment, median tumour length, esophageal tumour location, and tumour stage. Events and toxicity related to treatment were also included in the computer data base.

Tumour stage

The 1983 AJCC staging system was used in this study according to recently published recommendations[21]. Tumour evaluation was based on oesophagoscopy, barium oesophagography, chest and abdominal computed tomography (CT-scan), endoscopic bronchoscopy and esophageal ultrasonography when feasible.

Treatment schedule

CRT regimen was based on the 5 FU/CDDP CT combination associated with an external RT. The RT was delivered either by a dose of 50 Gy (50 Gy/25 fractions per 5 wk) with concomitant CT courses delivered on wk 1 and 5, or either a dose of 60 Gy (20 Gy/10 fractions × 3 courses separated by a 2-wk break) with concomitant CT courses delivered on wk 1, 5 and 9. The CT courses combined 5-FU (750 to 1000 mg/m^2 per day delivered by continuous infusion on 4 d) and CDDP (75 to 100 mg/m^2 delivered on 1 d). The target volume of RT was the macroscopic tumour and enlarged lymph nodes, if any, surrounded by 5 cm proximal and distal margins and a 2 cm radial margin. The target was extended to the inferior cervical area in cases of tumours located above the carina. The specified dose was delivered at the intersection of the central axis of the beams, according to international guidelines. The irradiation technique was applied in anterior and posterior opposed fields. At 40 Gy, the radiation portals were reduced to shield the spinal cord and encompass the primary tumour with a 2-3 cm craniocaudal margin.

Evaluation of clinical response and toxicity to CRT

Patients were considered to have a clinical complete response (CCR) to CRT when no residual tumour was identified on endoscopy and when no metastatic disease occurrence was observed on CT-scan. This evaluation was performed 2 mo after CRT completion.

Toxicity related to the treatment was evaluated using the National Cancer Institute Common Toxicity Criteria (NCI-CTC, version 2.0). Toxicity was assessed in each patient at d 1 of each chemotherapy course. At each course, patients received the treatment when they exhibited a WHO performance status of 2 or less; satisfactory haematologic function (leucocytes count $\geqslant$ 3000 mm^{-3}, platelet count $\geqslant$ 100 000 mm^{-3}) and good renal function (creatine serum level $\leqslant$ 100 micromole/L). Patients with major complication i.e. heart disease, pulmonary fibrosis, or active carcinoma at the other site were not eligible for treatment.

Follow-up

The follow-up was performed on clinical basis, endoscopy and CT scan. Histopathological confirmation of the recurrence was not routinely required. Follow-up was performed either until death or for the purpose of this study until October 2005.

Statistical analysis

Analysis was performed in October 2005 and was considered the cut-off date. Survival curves according to the putative prognostic factors were established using the Kaplan-Meier method and were compared with a log-rank test. The effects of clinical characteristics at baseline related to prognosis using univariate analysis were further evaluated in multivariate analysis using Cox regression model. A two-side *P*-value equal or less than 0.05 was considered to indicate statistical significance. Data from patients who had been lost to follow-up were censored at the time of last obtained information. The date of CRT initiation was the starting point for the analysis of overall survival. The date of CRT response evaluation was the starting point for the analysis of the disease free survival.

RESULTS

Patients characteristics

Between the first of January 1994 and the 31st December 2000, one hundred and sixteen consecutive patients were treated with a CRT based on the 5FU/CDDP CT combination. The majority of patients had a good performance status and the dysphagia score prior to CRT reflected their ability to eat a normal or semisolids diet for approximately 90% of these patients (Table 1). Among patients who were estimated with T1-T2 tumour on CT-scan, 14 were estimated to present with a T1-T2 N0 tumour. These latter patients were treated with a definitive CRT as regards age and/or comorbidities.

Safety and toxicity per patient

Significant toxicities per patient are shown in Table 2.

Table 1 Patient characteristics

	n = 116	%
Mean age (yr)	61.3 (40-90)	
Male	101	87.1
Female	15	12.9
WHO performance status (OMS)		
0	32	27.6
1	70	60.3
2	14	12.1
Dysphagia (Atkinson score)		
0	2	1.7
1	21	18.1
2	58	50
3	26	22.4
4	9	7.8
Weight loss ⩾ 10% at CRT		
Initiation	30	25.9
TNM		
T 1-2	27	23.3
3-4	89	76.7
N 0	50	43.1
1	54	46.5
x	12	10.4
M 0	116	100
1	-	-
Esophageal location		
Upper one-third	35	30.2
Middle one-third	53	45.7
Lower one third	28	24.1
Mean tumour length (cm)	4.9 (0-15)	
Histopathology		
Squamous cell carcinoma	116	

Table 2 Significant treatment toxicities per patient (%)

	Grade 3 (%)	Grade 4 (%)
Haematological		
Neutropenia	9	3
Anemia	11	-
Thrombopenia	1	1
Mucositis		
Oral	4	-
Esophageal	16	-
Gastrointestinal		
Nausea	12	-
Diarrhoea	4	-
Neuropathy	1	-
Alopecia	3	-

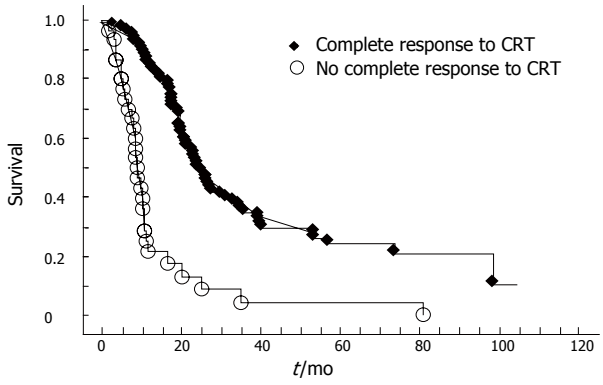

Figure 1 Survival according to response to CRT. The median overall survival of patients who had a complete clinical response (CCR) to the chemoradiotherapy (CRT) was 25 mo as compared to 9 mo in non-responder patients ($P < 0.001$).

There was no death related to CRT. Moreover, 75 patients (64.6%) experienced grade 3-4 toxicities and 112 (96.5%) patients achieved the planned concomitant CRT regimen. Dose modification to the planned CT regimen were required in 55 patients (47.4%) and 18 patients (15.5%) received at least one of their CT courses with a delay of more than 1 wk. The mean delivered radiation dose was 53.2 Gy.

During the CRT treatment, 18 patients (15.5%) required nutritional enteral feeding. In contrast, palliation of the dysphagia by endoscopic stenosis dilation was performed in 25 patients (21.6%). A self expandable metallic stent was inserted in 6 patients (5.1%) during CRT treatment.

Clinical complete response (CCR) to CRT

A total of 86/116 patients (74.1%) achieved a CCR to CRT. In the remaining 30 non-responder patients, a self expanding metallic stent was inserted in 12 for dysphagia palliation after completion of CRT, a CT treatment was initiated in 1 patient and salvage surgery was performed in 2 patients.

Patient outcome

In October 2005, 7/116 patients (6%) were still alive. The median follow-up of these surviving patients was 79 mo (range 56-104) and the median follow-up of the 11 patients who were lost to follow-up during the study was 45.1 mo (range 9-68).

The median overall survival was 20 mo (range 2-114) and the 2-years and 5-years survival rates were 39.6% and 9.4%, respectively. Moreover, the median overall survival of the 86 patients who had CCR to CRT was 25 mo (range 3-114) as compared to 9 mo (range 2-81) in non-responder patients ($P < 0.001$) (Figure 1). The median disease free survival of responder patients to CRT was 17 mo.

During the follow-up, 34 of responder patients (39.5%) experienced a local disease recurrence, 37 patients (43%) experienced metastatic disease and 19 of them experienced both of these recurrences.

Prognostic factors of survival

As regards univariate analysis (Table 3), survival was correlated to CCR to CRT ($P < 0.001$), WHO performance status < 2 ($P = 0.01$) and tumour length < 6 cm ($P = 0.045$). In contrast, weight loss > 10% at the start of CRT was in limit of statistical significance and was included in multivariate analysis ($P = 0.053$). In a Cox regression model (Table 3), the independent covariates significantly associated with survival were the CCR to CRT ($P < 0.0001$; Odds Ratio (OR): 0.121; IC95 = 0.06-0.24), the weight loss < 10% ($P = 0.034$; OR: 0.53; IC95 = 0.29-0.95) and a WHO performance status < 2 ($P = 0.046$; OR: 0.495; IC95 = 0.24-0.99).

DISCUSSION

To date, definitive CRT based on the 5FU/CDDP combination is considered as standard treatment in non operable patients with locally advanced esophageal carcinoma whatever the histological tumour phenotype[3-5]. Some authors recently suggested that histological types of oesophagus tumour could be considered separately regarding their significant different treatment response and long term prognosis[9-11]. Therefore, we performed a retrospective analysis of the long term outcome and predictive factors of survival in 116 patients with LASCOC treated with a definitive CRT using the 5FU/CDDP CT combination.

In our study, the 5-year survival was 9.4% and the median overall survival was 20 mo. We also found that responder patients to CRT had a significantly increased median survival as compared to non-responders patients (24 mo *vs* 9 mo; $P < 0.001$). This result was supported by the multivariate analysis which identified the CCR as an independent prognostic factor of survival. In definitive CRT series using the 5FU/CDDP combination, a survey of literature showed that median overall survival ranged from 17 to 26 mo and the 5-year survival rate from 20% to 30%[12-20]. The 5-year survival rate in our study was slightly lower as compared to those reported in these series. This result could be explained by the patient selection bias in these prospective trials, whereas our retrospective study possibly reflected the outcome of non-selected patients with LASCOC treated with definitive RT.

The CCR to CRT was obtained in 75.9% of patients in our series. Moreover, a CCR was identified as an independent prognostic factor of long-term survival in our multivariate analysis. Although the prognostic significance of pathological complete response after preoperative CRT was well documented[22-24], to our knowledge, there are no previous studies that have reported similar result in patients with LASCOC treated with definitive CRT using the 5FU/CDDP combination. In fact, the significant impact of CCR to CRT in long-term survival in patients treated with the same definitive CRT regimen was reported in series which included patients with mixed histological tumour types[12,25,26]. Moreover, in the reported Ohtsu *et al* study focusing exclusively on patients with LASCOC, the CCR to CRT was identified as a predictive factor of the progression free survival but not for overall survival[18]. In our study, 39.5% of responder patients to CRT had a local disease recurrence. In previous studies, local recurrences were reported to be as high as 38% to 48% after definitive CRT[12,18]. Furthermore, a distant metastasis occurred in 43% of responder patients to CRT in our series. This result compared less favourably to other series including patients with squamous cell carcinoma treated with the same CT combination, where less than 30% of responder patients to CRT experienced a distant metastasis[12,18]. The frequent use of additional CT in responder patients to CRT in these latter series could probably explain the difference in metastasis frequency. Indeed, only 44% of patients received additional CT after the CRT completion in our study.

Thus, our results suggest that further optimisations of the definitive CRT regimen are required for both local and systemic disease control improvement. To date, the 5FU/CDDP combination used in our study is still considered the standard for the CRT regimen. Evaluation of novel chemotherapy regimens which include new drugs such as irinotecan and new cancer therapies encompassing those directed against vascular growth factor and epidermal growth factor pathways may be usefully associated with RT to provide improved sustained CCR and therefore optimal long-term survival[27-30]. Furthermore, CRT regimen optimisation should determine the optimal radiotherapy dose in order to achieve a sustained local disease control. In our study, patients received a mean dose of 52.1 Gy of external RT in the tumour bed, which was a similar dose as that used in the standard regimen described by Herskovic *et al*[3]. Recently, Zhang *et al* reported, in a retrospective study, that patient who received a dose of RT more than 51 Gy had a statistically better local disease-free survival and overall survival as compared to patients treated with a dose of RT less than 51 Gy[31]. Minsky *et al* specifically investigated dose escalation in RT. In this prospective study, patients were randomised in a CRT regimen using either 50.4 Gy of RT or 64.8 Gy of RT[32]. However, the unexplained excess of death frequency (9%) which occurred prior to the dose escalation in patients treated in the 64.8 Gy arm did not permit a valid conclusion as regards the optimal dose of RT. In our study, no death due to CRT was observed. However, we found that 64.6% of patient experienced grade 3-4 toxicities, which was a similar result to that usually reported[3,4]. As regards the new RT techniques including three dimensional CT planning with conformal beam, dose escalation in the definitive CRT regimen could be reconsidered. Further studies including these new RT techniques may be helpful to evaluate the optimal and safety dose of RT in patients with LASCOC.

Multivariate analysis identified the WHO performance status less than 2 as the second independent predictive factor of long-term survival in patients treated with definitive CRT for a LASCOC. The WHO performance status has been previously identified as prognostic factor in patients with esophageal cancer treated by radiotherapy alone or in patients with metastatic oesophago-gastric cancer in previous studies[21,33]. Moreover, Polee *et al* also identified this variable as a prognostic factor of survival in

Table 3 Predictive factors of survival, univariate and multivariate analysis

	Univariate	Multivariate		
	P	*P*	OR	IC95
Sex	0.507	-		
Age < 70	0.745	-		
WHO performance status < 2	0.01	0.046	0.52	0.28-0.99
Weight loss < 10%	0.053	0.034	0.53	0.29-0.95
Dysphagia	0.074	-		
T	0.273	-		
N	0.499	-		
Tumour location	0.501	-		
Tumour length < 6 cm	0.045	0.534	0.86	0.53-1.38
Complete response to CRT	< 0.001	< 0.000	0.21	0.13-0.36

a meta-analysis performed in six prospective trials which included several CRT regimens and mixed histological type of esophageal tumour[34]. However, to our knowledge, this result had never been previously reported in patients with LASCOC treated in curative intent with definitive CRT using the 5FU/CDDP combination. The WHO performance status evaluation provides useful information on the patient's general well-being indirectly reflecting the impact of the malignancy on the physiological individual condition. Although the evaluation of the WHO performance status seems to be subjective, our results showed that this variable was significantly correlated with survival. Moreover, weight loss before treatment starting was also identified as prognostic factor in our work. This variable was recently to be correlated with prognosis in a meta-analysis by Thomas *et al* in patient treated by definitive CRT for esophageal cancer[30].

Interestingly, tumour characteristics were not identified as prognostic factors in our study. Indeed, the tumour diameter and the tumour length were only significant in univariate analysis. In surgical series, the extent of the tumour infiltration in the esophageal wall and the lymph node involvement were frequently closely correlated with survival[23,24,35]. Although an underpowered significance could not be excluded in the analysis, our results could also reflect the limited accuracy of the usual staging evaluation including oesophagography, oesophagoscopy and thoraco-abdominal computed tomography used during the period of the study. However, these imaging modalities appear to be more accurate for the CRT response assessment as regards the significant link that was observed between the CCR and survival. The high rate of early disease recurrence in our study could reflect an overestimation of the CCR rate by modalities used in our study. Thus, the further use of endoscopic ultrasound and PET-FDG, may be helpful to provide better correlative data for initial tumour staging and for tumour response assessment[36-38].

In conclusion, based on a large series of patients with LASCOC, our results suggest that survival of these patients treated with definitive CRT using the 5FU/ performance status. These prognostic factors could be considered for the management of individual patients as well as a stratification variable for the design and interpretation of further randomised trials. However, as regards the retrospective design of our study, further prospective studies are necessary to investigate the impact of these prognostic factors.

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REFERENCES

1 **Keighley MR**. Gastrointestinal cancers in Europe. *Aliment Pharmacol Ther* 2003; **18** Suppl 3: 7-30
2 **Ménégoz F**, Black RJ, Arveux P, Magne V, Ferlay J, Buémi A, Carli PM, Chapelain G, Faivre J, Gignoux M, Grosclaude P, Mace-Lesec'h J, Raverdy N, Schaffer P. Cancer incidence and mortality in France in 1975-95. *Eur J Cancer Prev* 1997; **6**: 442-466
3 **Herskovic A**, Martz K, al-Sarraf M, Leichman L, Brindle J, Vaitkevicius V, Cooper J, Byhardt R, Davis L, Emami B. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *N Engl J Med* 1992; **326**: 1593-1598
4 **Cooper JS,** Guo MD, Herskovic A, Macdonald JS, Martenson JA Jr, Al-Sarraf M, Byhardt R, Russell AH, Beitler JJ, Spencer S, Asbell SO, Graham MV, Leichman LL. Chemoradiotherapy of locally advanced esophageal cancer: long-term follow-up of a prospective randomized trial (RTOG 85-01). Radiation Therapy Oncology Group. *JAMA* 1999; **281**: 1623-1627
5 **Bedenne L,** Michel P, Bouche O, Triboulet JP, Conroy T, Pezet D, Roullet B, Seitz JF, Lacourt J, Milan C. Randomized phase III trial in locally advanced esophageal cancer: radiochemotherapy followed by surgery versus radiochemotherapy alone (FFCD 9102). *Proc Am Soc Clin Oncol* 2002: 519a
6 **Stahl M,** Stuschke M, Lehmann N, Meyer HJ, Walz MK, Seeber S, Klump B, Budach W, Teichmann R, Schmitt M, Schmitt G, Franke C, Wilke H. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. *J Clin Oncol* 2005; **23**: 2310-2317
7 **Burmeister BH,** Denham JW, O'Brien M, Jamieson GG, Gill PG, Devitt P, Yeoh E, Hamilton CS, Ackland SP, Lamb DS. Combined modality therapy for esophageal carcinoma: preliminary results from a large Australasian multicenter study. *Int J Radiat Oncol Biol Phys 1995*; **32**: 997-1006
8 **Chan A**, Wong A. Is combined chemotherapy and radiation therapy equally effective as surgical resection in localized esophageal carcinoma? *Int J Radiat Oncol Biol Phys* 1999; **45**: 265-270
9 **Terry P,** Lagergren J, Ye W, Nyrén O, Wolk A. Antioxidants and cancers of the esophagus and gastric cardia. *Int J Cancer* 2000; **87**: 750-754
10 **Enzinger PC,** Mayer RJ. Esophageal cancer. *N Engl J Med* 2003; **349**: 2241-2252
11 **Rizk NP,** Bains MS, Ilson DH, Minsky B, Rush VW. The AJCC Staging System Does Not Predict Survival in Patients Receiving Multimodality Therapy for Esophageal Cancer. *Proc Am Soc Clin Oncol* 2005: 4005a
12 **Seitz JF**, Milan C, Giovannini M, Dumas F, Cauvin JM, Conroy T, François E, Renard P, Votte-Lambert A, Paillot B, Bedenne L. [Concurrent concentrated radio-chemotherapy of epidermoid cancer of the esophagus. Long-term results of a phase II national multicenter trial in 122 non-operable patients (FFCD 8803)]. *Gastroenterol Clin Biol* 2000; **24**: 201-210
13 **Leichman L,** Herskovic A, Leichman CG, Lattin PB, Steiger Z, Tapazoglou E, Rosenberg JC, Arbulu A, Asfaw I, Kinzie J. Nonoperative therapy for squamous-cell cancer of the esophagus. *J Clin Oncol* 1987; **5**: 365-370
14 **Seitz JF,** Giovannini M, Padaut-Cesana J, Fuentes P, Giudicelli R, Gauthier AP, Carcassonne Y. Inoperable nonmetastatic squamous cell carcinoma of the esophagus managed by concomitant chemotherapy (5-fluorouracil and cisplatin) and radiation therapy. *Cancer* 1990**; 66**: 214-219
15 **Reddy SP,** Lad T, Mullane M, Rosen F, Carroll R, Marks JE. Radiotherapy alone compared with radiotherapy and chemotherapy in patients with squamous cell carcinoma of the esophagus. *Am J Clin Oncol* 1995; **18**: 376-381
16 **Murakami M,** Kuroda Y, Okamoto Y, Kono K, Yoden E, Kusumi F, Hajiro K, Matsusue S, Takeda H. Neoadjuvant concurrent chemoradiotherapy followed by definitive high-dose radiotherapy or surgery for operable thoracic esophageal carcinoma. *Int J Radiat Oncol Biol Phys* 1998; **40**: 1049-1059
17 **Minsky BD,** Neuberg D, Kelsen DP, Pisansky TM, Ginsberg RJ, Pajak T, Salter M, Benson AB 3rd. Final report of Intergroup Trial 0122 (ECOG PE-289, RTOG 90-12): Phase II trial of neoadjuvant chemotherapy plus concurrent chemotherapy and high-dose radiation for squamous cell carcinoma of the esophagus. *Int J Radiat Oncol Biol Phys* 1999; **43**: 517-523
18 **Ohtsu A,** Boku N, Muro K, Chin K, Muto M, Yoshida S, Satake M, Ishikura S, Ogino T, Miyata Y, Seki S, Kaneko K,

Nakamura A. Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. *J Clin Oncol* 1999; **17**: 2915-2921

19 **Hironaka S**, Ohtsu A, Boku N, Muto M, Nagashima F, Saito H, Yoshida S, Nishimura M, Haruno M, Ishikura S, Ogino T, Yamamoto S, Ochiai A. Nonrandomized comparison between definitive chemoradiotherapy and radical surgery in patients with T(2-3)N(any) M(0) squamous cell carcinoma of the esophagus. *Int J Radiat Oncol Biol Phys* 2003; **57**: 425-433

20 **Kaneko K,** Ito H, Konishi K, Kurahashi T, Ito T, Katagiri A, Yamamoto T, Kitahara T, Mizutani Y, Ohtsu A, Mitamura K. Definitive chemoradiotherapy for patients with malignant stricture due to T3 or T4 squamous cell carcinoma of the oesophagus. *Br J Cancer* 2003; **88**: 18-24

21 **Coia LR**, Minsky BD, Berkey BA, John MJ, Haller D, Landry J, Pisansky TM, Willett CG, Hoffman JP, Owen JB, Hanks GE. Outcome of patients receiving radiation for cancer of the esophagus: results of the 1992-1994 Patterns of Care Study. *J Clin Oncol* 2000; **18**: 455-462

22 **Forastiere AA,** Orringer MB, Perez-Tamayo C, Urba SG, Zahurak M. Preoperative chemoradiation followed by transhiatal esophagectomy for carcinoma of the esophagus: final report. *J Clin Oncol* 1993; **11**: 1118-1123

23 **Fujita H,** Sueyoshi S, Tanaka T, Tanaka Y, Sasahara H, Shirouzu K, Suzuki G, Hayabuchi N, Inutsuka H. Prospective non-randomized trial comparing esophagectomy-followed-by-chemoradiotherapy versus chemoradiotherapy-followed-by-esophagectomy for T4 esophageal cancers. *J Surg Oncol* 2005; **90**: 209-219

24 **Berger AC,** Farma J, Scott WJ, Freedman G, Weiner L, Cheng JD, Wang H, Goldberg M. Complete response to neoadjuvant chemoradiotherapy in esophageal carcinoma is associated with significantly improved survival. *J Clin Oncol 2005*; **23**: 4330-4337

25 **Lim JT,** Truong PT, Berthelet E, Pai H, Joe H, Wai E, Larsson S, Kader HA, Weinerman B, Wilson K, Olivotto IA. Endoscopic response predicts for survival and organ preservation after primary chemoradiotherapy for esophageal cancer. *Int J Radiat Oncol Biol Phys* 2003; **57**: 1328-1335

26 **Tahara M,** Ohtsu A, Hironaka S, Boku N, Ishikura S, Miyata Y, Ogino T, Yoshida S. Clinical impact of criteria for complete response (CR) of primary site to treatment of esophageal cancer. *Jpn J Clin Oncol* 2005; **35**: 316-323

27 **Ilson DH**, Forastiere A, Arquette M, Costa F, Heelan R, Huang Y, Kelsen DP. A phase II trial of paclitaxel and cisplatin in patients with advanced carcinoma of the esophagus. *Cancer J* 2000; **6**: 316-323

28 **Ajani JA**, Walsh G, Komaki R, Morris J, Swisher SG, Putnam JB Jr, Lynch PM, Wu TT, Smythe R, Vaporciyan A, Faust J, Cohen DS, Nivers R, Roth JA. Preoperative induction of CPT-11 and cisplatin chemotherapy followed by chemoradiotherapy in patients with locoregional carcinoma of the esophagus or gastroesophageal junction. *Cancer* 2004; **100**: 2347-2354

29 **Michel P,** Mirabel X, Galais M, Dahan L, Conroy T, Boucher E, Oprea C, Paillot B. Weekly cisplatin/irinotecan and radiotherapy (RT) in patients with locally advanced esophageal cancer : multicenter FFCD phase II trial. *Proc Am Soc Clin Oncol* 2005: 4060a

30 **Thomas CR Jr,** Berkey BA, Minsky BD, Gaspar LE, Herskovic A, Rich TA, Gunderson LL. Recursive partitioning analysis of pretreatment variables of 416 patients with locoregional esophageal cancer treated with definitive concomitant chemoradiotherapy on Intergroup and Radiation Therapy Oncology Group trials. *Int J Radiat Oncol Biol Phys* 2004; **58**: 1405-1410

31 **Zhang Z,** Liao Z, Jin J, Ajani J, Chang JY, Jeter M, Guerrero T, Stevens CW, Swisher S, Ho L, Yao J, Allen P, Cox JD, Komaki R. Dose-response relationship in locoregional control for patients with stage II-III esophageal cancer treated with concurrent chemotherapy and radiotherapy. *Int J Radiat Oncol Biol Phys* 2005; **61**: 656-664

32 **Minsky BD,** Pajak TF, Ginsberg RJ, Pisansky TM, Martenson J, Komaki R, Okawara G, Rosenthal SA, Kelsen DP. INT 0123 (Radiation Therapy Oncology Group 94-05) phase III trial of combined-modality therapy for esophageal cancer: high-dose versus standard-dose radiation therapy. *J Clin Oncol* 2002; **20**: 1167-1174

33 **Chau I,** Norman AR, Cunningham D, Waters JS, Oates J, Ross PJ. Multivariate prognostic factor analysis in locally advanced and metastatic esophago-gastric cancer--pooled analysis from three multicenter, randomized, controlled trials using individual patient data. *J Clin Oncol* 2004; **22**: 2395-2403

34 **Polee MB**, Hop WC, Kok TC, Eskens FA, van der Burg ME, Splinter TA, Siersema PD, Tilanus HW, Stoter G, van der Gaast A. Prognostic factors for survival in patients with advanced oesophageal cancer treated with cisplatin-based combination chemotherapy. *Br J Cancer* 2003; **89**: 2045-2050

35 **Kato H**, Miyazaki T, Nakajima M, Takita J, Kimura H, Faried A, Sohda M, Fukai Y, Masuda N, Fukuchi M, Manda R, Ojima H, Tsukada K, Kuwano H, Oriuchi N, Endo K. The incremental effect of positron emission tomography on diagnostic accuracy in the initial staging of esophageal carcinoma. *Cancer* 2005; **103**: 148-156

36 **Flamen P,** Van Cutsem E, Lerut A, Cambier JP, Haustermans K, Bormans G, De Leyn P, Van Raemdonck D, De Wever W, Ectors N, Maes A, Mortelmans L. Positron emission tomography for assessment of the response to induction radiochemotherapy in locally advanced oesophageal cancer. *Ann Oncol* 2002; **13**: 361-368

37 **Wu LF,** Wang BZ, Feng JL, Cheng WR, Liu GR, Xu XH, Zheng ZC. Preoperative TN staging of esophageal cancer: comparison of miniprobe ultrasonography, spiral CT and MRI. *World J Gastroenterol* 2003; **9**: 219-224

38 **Rice TW.** Clinical staging of esophageal carcinoma. CT, EUS, and PET. *Chest Surg Clin N Am* 2000; **10**: 471-485

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Pegylated-interferon alpha 2a treatment for chronic hepatitis C in patients on chronic haemodialysis

Ioan Sporea, Alina Popescu, Roxana Şirli, Ovidiu Golea, Camelia Totolici, Mirela Dănilă, Corina Vernic

Ioan Sporea, Alina Popescu, Roxana Şirli, Mirela Dănilă, Department of Gastroenterology and Hepatology, University of Medicine and Pharmacy "Victor Babeş" Timişoara, Romania
Ovidiu Golea, Camelia Totolici, Department of Haemodialysis and Renal Transplantation, County Hospital Timişoara, Romania
Corina Vernic, Department of Medical Informatics, University of Medicine and Pharmacy "Victor Babeş" Timişoara, Romania
Correspondence to: Professor Ioan Sporea, University of Medicine and Pharmacy, Department of Gastroenterology, 156, Iosif Bulbuca str. 300736 Timisoara, Romania. isporea@excite.com
Telephone: +40-256-309455 Fax: +40-256-309455
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Abstract

AIM: To evaluate the response to pegylated-interferon alpha 2a in chronic hepatitis C patients on chronic haemodialysis.

METHODS: Ten patients with chronic C hepatitis were enrolled in this study. All had increased aminotransferases for more than 6 mo, positive antiHCV antibodies and positive PCR HCV-RNA. We administrated Peg-Interferon alpha 2a 180 μg/wk for 48 wk. After 12 wk of treatment we evaluated the biochemical and early virological response (EVR). At the end of the treatment we evaluated the biochemical response and 24 wk after the end of the treatment we evaluated the sustained virological response (SVR). We monitored the side-effects during the treatment.

RESULTS: Two patients dropped out in the first 12 wk of treatment and 2 after the first 12 wk of treatment. After 12 wk of treatment, 7 out of 8 patients had biochemical response and EVR and 1 had biochemical response but persistent viremia. We had to reduce the dose of pegylated-interferon to 135 μg/wk in 2 cases. Three out of 6 (50%) patients had SVR 24 wk after the end of the treatment. Intention-to-treat analysis showed that 3 out of 10 patients (30%) had SVR. Side-effects occurred in most of the patients (flu-like syndrome, thrombocytopenia or leucopoenia), but they did not impose the discontinuation of treatment.

CONCLUSION: After 12 wk of treatment with Peg-Interferon alpha 2a (40 ku) in patients on chronic haemodialysis with chronic C hepatitis, EVR was obtained in 87.5% (7/8) of the cases. SVR was achieved in 50% of the cases (3/6 patients) that finished the 48 wk of treatment.

INTRODUCTION

Although constant efforts have been made to improve the outcome of hepatitis C patients, chronic infection with hepatitis C virus (HCV) remains a problem for hepatologists. The development of new therapeutic formulas (pegylation) and the introduction of ribavirin were major steps forward. However the problem is not entirely solved since the sustained virological response can be obtained in only half of HCV-infected patients. There are also the special groups of patients (with liver cirrhosis, with HIV coinfection, patients on chronic haemodialysis) in which the optimal antiviral treatment is still not established.

In patients on chronic haemodialysis, the number of individuals infected with HCV is rather high mostly due to nosocomial infection. The reported prevalence of HCV infection ranges from 8% to 20% in dialysis patients in developed countries[1-5] and much higher in less developed countries[6]. The prevalence of anti-HCV among dialysis patients was 43.9% in Saudi Arabia in 2001[7], 30% in India in 2002[8], and 41% in Turkey (2001)[9]. In United States of America in 2000, 8.4% of haemodialysis patients were anti-HCV positive[5]. The incidence of HCV infection is higher in patients undergoing dialysis at hospitals than in those undergoing haemodialysis or peritoneal dialysis at home.

The main mechanisms involved in nosocomial infection with HCV in haemodialysis patients are filter reuse, use of contamined haemodialysis machines and contamination of medical staff's hands[10]. It has been proven that the incidence of HCV infection in haemodialysis patients increases if the nurse does not change her gloves before injecting each patient[11] and if HCV (+) patients undergo haemodialysis in the same room with HCV (-) patients[12].

Other possible risk factors for transmission of the virus are sharing single vials to prepare drugs or infusions for different patients, distance less then one meter between chairs[13], sharing a single heparin-saline solution ampoule in different patients[14]. A large French multi-center study[15] on 1323 haemodialyzed patients has shown an incidence of 0.4% new HCV infections per year, almost two thirds of them occurring in infected patients on dialysis during the same shift at the same unit.

Regardless of the route of infection, the evolution of HCV-infected patients on chronic haemodialysis is often severe. Martin *et al*[16] showed that 24% of haemodialysis patients with positive anti-HCV Ab have liver cirrhosis and that there is no correlation between the severity of hepatic lesions and viral genotype, viral load or transaminase level. Hence we must treat chronic hepatitis C in haemodialysis patients, particularly in those on the waiting list for renal transplantation, because post-transplant immunosuppressive therapy can accelerate the natural course of the liver disease. Interferon-based therapy is not recommended in HCV positive patients after renal transplantation due to a significant risk of graft loss and a low rate of clearance of the virus[17,18]. Also ribavirin monotherapy for renal transplant recipients positive for anti-HCV is associated with improvement in liver enzymes but not significant change of HCV RNA[19]. On the other hand, Kamar *et al*[20] showed that treatment of HCV positive haemodialysis patients with interferon α could induce complete and sustained clearance of the virus in almost 29% of them, without any relapses after renal transplantation despite subsequent immunosuppressive treatment.

Another problem of the treatment for HCV-infected patients on dialysis is the contraindication of ribavirin, due to the risk of deep and long-lasting haemolytic anaemia[21].

Due to these characteristics of this special group of patients and the promising results of our previous study using standard interferon in haemodialysis patients[22], we decided to evaluate the effect of pegylated-interferon in patients with chronic hepatitis C on dialysis.

MATERIALS AND METHODS

We included 10 haemodialysis patients in our study (4 males and 6 females, mean age 40.2 years). Written informed consent to participate in this study was obtained from all of them. All had increased aminotransferases for more than 6 mo, anti-HCV antibodies (Elisa Ⅲ) and positive PCR HCV-RNA. The viral load at admission and after 12 wk of treatment (EVR) was determined by the classical polymerase chain reaction (Roche) with a detection limit of 600 UI/mL. The viral load 24 wk after the end of treatment (SVR) was determined by real-time PCR (Abbott) with a detection limit of 23 UI/mL. None of the patients presented with clinical, biological, endoscopic or ultrasound signs of liver cirrhosis. We did not perform liver biopsy because of the increased risk of bleeding in haemodialysis patients.

All patients were treated with pegylated-interferon alpha 2a (180 μg/wk) for 48 wk. We evaluated the biochemical response every month and the virological response after 12 wk of treatment (early virological response-EVR) and 24 wk after the end of treatment.

We monitored the side effects during the treatment. At the end of the treatment we evaluated the biochemical response of our patients (number of patients with normal transaminases) and the sustained virological response (SVR) 24 wk after the end of the treatment (72 wk from the beginning of the treatment) by determining the virological load.

RESULTS

The 10 patients studied are listed in Table 1. At the beginning of the study the virological load was low in 2 patients (< 10 kIU/mL), moderate in 5 patients (10-500 kIU/mL), and high in 3 patients (> 500 kIU/mL). Two patients were excluded from the study. One patient was excluded because of lack of compliance and 1 patient discontinued the treatment due to complications after surgery (sepsis).

We determined the biochemical and virological response (PCR RNA HCV) in the 8 patients who continued the treatment after 12 wk of therapy. Of these patients, 7 (87.5%) had biochemical response (normal transaminases) as well as virological response (viral load < 0.6 kIU/mL), 1 (12.5%) had biochemical response (normal transaminases) but persistent viremia. We continued the treatment with pegylated-interferon alpha 2a for 48 wk. During this period one patient died of cerebral haemorrhage caused by arterial hypertension after 16 wk of therapy (the patient having normal prothrombin time and only mild thrombocytopenia-104 000 platelets/mL) and one patient was excluded from the study due to lack of compliance after 28 wk of therapy.

The total number of patients who finished the 48-wk treatment was 6 (60%). All of them had biochemical response at the end of treatment (normal transaminases). Three out of 6 patients (50%) had sustained virological response (SVR) 24 wk after the end of the treatment. The intention-to-treat analysis showed that 3 out of 10 patients (30%) had sustained virological response 24 wk after the end of the treatment.

All patients had minor flue-like symptoms, 4 had mild thrombocytopenia (Tr < 150 000/mm^3) and 2 had moderate thrombocytopenia (Tr < 100 000/mm^3), 4 had transitory mild leucopoenia (L < 4000/mm^3). In the 6th mo of therapy one of the patients developed sepsis secondary to central venous catheter infection. During this period the patient had elevated transaminases. Unfortunately, this patient abandoned the treatment one month later.

We modified the dose of pegylated-interferon in 2 patients. In one we reduced it to 135 μg/wk for 1 mo (because of the thrombocytopenia and haemorrhagic complications-metroragia, epistaxis, prolonged bleeding of the fistula), then 180 μg/wk was administered again. In the second patient the dose reduction to 135 μg/wk was initiated in the 4th mo of therapy until the end of 48-wk treatment.

We did not stop the treatment in any patient due to severe side effects of pegylated- interferon.

Table 1 Demographic data of haemodialysis patients treated with pegylated interferon

No.	Patient	Sex	Age	Duration of treatment wk	Stop of because treatment of	Initial viral load (UI/mL)	Viral load at wk 12 (UI/mL)	Viral load at wk 72 (UI/mL)
1	B.L.	F	42	48		4090	< 600	< 23
2	C.A.	M	34	48		84 760	< 600	45
3	B.V.	M	45	48		252 000	< 600	263 300
4	L.D.	F	40	6	Non compliance	8000	-	-
5	B.L.	F	44	48		157 000	< 600	< 23
6	P.E.	F	28	48		+	< 600	< 23
7	C.M.	F	47	48		766 000	60 100	178
8	C.A.	F	45	8	Complication after surgery	417 000	-	-
9	T.M.	M	26	16	Death of cerebral haemorrhage	> 1 000 000	< 600	-
10	P.C.	M	51	28	Non compliance	> 1 000 000	< 600	-

DISCUSSION

Many clinical trials have focused on the treatment of chronic hepatitis C patients on chronic haemodialysis with standard interferon, because ribavirin is not recommended. Some studies have used ribavirin at low doses (170-300 mg/d) together with standard interferon[23]. The results are encouraging but a careful monitoring of anaemia is mandatory. When anaemia occurs it is corrected with high doses of erythropoetin. On the other hand, post-transplant treatment of chronic C hepatitis with interferon is not recommended because it can induce graft rejection (15.4%-63.6% of cases)[24]. Also, post-transplant monotherapy with ribavirin or amantadine has been proven inefficient[24].

Fabrizi *et al*[6] have found a mean SVR of 37% and a mean dropout rate of 17% in chronic hepatitis C patients on dialysis after interferon monotherapy. Our experience in treatment of these patients with standard interferon showed that sustained biochemical response is 46.1% and sustained virological response (HCV-RNA) is 38.4% respectively 6 mo after interferon treatment[22].

The promising results of monotherapy with standard interferon in chronic haemodialysis patients with chronic hepatitis C[22,25-27], have shown that viral clearance occurs in 27%[27] to 64%[26] of patients after 12 mo of treatment with standard interfreon.

In patients on chronic haemodialysis, the combined treatment with interferon and ribavirin is difficult to manage because haemolysis is induced by ribavirin. There are studies in which ribavirin is administrated at low doses (170-300 mg/d), the results are remarkable but anaemia should be carefully monitored[23].

The second therapeutic option for patients on chronic haemodialysis with chronic C hepatitis is to use pegylated interferon. In most of the studies performed in patients with chronic C hepatitis and normal renal function, the response rate doubled when the patients switched from standard interferon to pegylated-interferon. Some 3 rd phase studies have been performed in Greece, Mexico, Great Britain and USA to evaluate the sustained virological response after treatment with pegylated interferon alpha 2a in patients on chronic haemodialysis.

Martin *et al*[28] demonstrated that the absorption, distribution and total clearance of pegylated-interferon alpha 2a (40 ku) are not very different from those in patients with normal renal function, and that the tolerability of pegylated-interferon alpha 2a and the adverse effects in patients on chronic haemodialysis are similar with those in patients without renal impairment. In our group the side effects were quite the same with those in "normal" patients with chronic hepatitis C treated with pegylated-interferon.

We reduced the dose of pegylated-interferon to 135 μg/wk in 2 cases (in one patient only for one month and in another until the end point of treatment). Various authors have recommended a dose of 180 or 135 μg/wk of pegylated-interferon alpha 2a in patients on chronic haemodialysis. We prefer to start with 180 μg/wk in order to reduce the dosage if severe side effects occur. We reduced the dosage in 2 patients due to thrombocytopenia and bleeding.

Data on the patients on haemodialysis treated with pegylated-interferon alpha 2b are rather discouraging. A study by Russo *et al*[29] on the HCV-infected patients on haemodialysis treated with pegylated-interferon alpha-2b showed that a poor tolerance is associated with substantial side effects. Also, a case report by Potthoff *et al*[30] showed that IFN-alpha 2b three times a week after haemodialysis seems to be better tolerated than pegylated-interferon-alpha 2b once a week. A randomized study performed by Mahmoud *et al*[31] in pretransplant haemodialysis patients with chronic hepatitis C treated with standard interferon alpha 2b, showed that IFN-treated patients have significantly better post-transplant hepatic functions and significantly lower rates of chronic allograft nephropathy. Further studies are needed to find out which type of interferon is better tolerated and has better results for the treatment of haemodialyzed patients with chronic hepatitis C.

Since there are more and more encouraging results of treatment with interferon in patients on chronic haemodialysis with chronic hepatitis C, it is likely that very soon all these patients can benefit from antiviral therapy (standard interferon alone or in combination with ribavirin, or pegylated-interferon).

After 12 wk of treatment with Peg-Interferon alpha 2a (40 ku) in patients on chronic haemodialysis with chronic C hepatitis, the early virological response (EVR) (HCV-RNA absent by PCR) was obtained in 87.5% (7/8) of the cases. All the patients that finished the 48 wk of treatment had normal transaminases (biochemical response) (6/6). We had to reduce the dose of Peg-Interferon in only 2 cases. Even if side effects occurred in most of the patients

(flue-like syndrome, thrombocytopenia or leucopoenia) they did not impose the discontinuation of treatment. The sustained virological response at 6 mo after the end of the therapy was achieved in 50% of the cases (3/6 patients) that finished the course of 48 wk of treatment.

REFERENCES

1 **Lombardi M**, Cerrai T, Geatti S, Negroni S, Pertusini L, Pegoraro M, Di Lullo G. Results of a national epidemiological investigation on HCV infection among dialysis patients. (Survey by the Italian Branch of EDTNA/ERCA). *J Nephrol* 1999; **12**: 322-327

2 **Salama G**, Rostaing L, Sandres K, Izopet J. Hepatitis C virus infection in French hemodialysis units: a multicenter study. *J Med Virol* 2000; **61**: 44-51

3 **Jadoul M**, Cornu C, van Ypersele de Strihou C. Universal precautions prevent hepatitis C virus transmission: a 54 month follow-up of the Belgian Multicenter Study. The Universitaires Cliniques St-Luc (UCL) Collaborative Group. *Kidney Int* 1998; **53**: 1022-1025

4 **Schneeberger PM**, Keur I, van der Vliet W, van Hoek K, Boswijk H, van Loon AM, van Dijk WC, Kauffmann RH, Quint W, van Doorn LJ. Hepatitis C virus infections in dialysis centers in The Netherlands: a national survey by serological and molecular methods. *J Clin Microbiol* 1998; **36**: 1711-1715

5 **Tokars JI**, Frank M, Alter MJ, Arduino MJ. National surveillance of dialysis-associated diseases in the United States, 2000. *Semin Dial* 2002; **15**: 162-171

6 **Fabrizi F**, Dulai G, Dixit V, Bunnapradist S, Martin P. Meta-analysis: interferon for the treatment of chronic hepatitis C in dialysis patients. *Aliment Pharmacol Ther* 2003; **18**: 1071-1081

7 **Saxena AK**, Panhotra BR, Naguib M, Aboras MN, Sundaram DS, Venkateshappa CK, Khan WU. Prevalence of hepatitis C antibodies among hemodialysis patients in Al-hasa region of saudi arabia. *Saudi J Kidney Dis Transpl* 2001; **12**: 562-565

8 **Jaiswal SK**, Chitnis DS, Salgia P, Sepaha A, Pandit CS. Prevalence of hepatitis viruses among chronic renal failure patients on haemodialysis in Central India. *Dial Transplant* 2002; **31**: 234-240

9 **Yilmaz ME**, Kara IH, Sari Y, Duzen S, Usul Y, Isikoglu B. Seroprevalence and risk factors of HCV in dialysis patients in a university haemodialysis center of southeast Anatolia, Turkey. *Dial Transplant* 2001; **30**: 748-755

10 **Fabrizi F**, Poordad FF, Martin P. Hepatitis C infection and the patient with end-stage renal disease. *Hepatology* 2002; **36**: 3-10

11 **Okuda K**, Hayashi H, Kobayashi S, Irie Y. Mode of hepatitis C infection not associated with blood transfusion among chronic hemodialysis patients. *J Hepatol* 1995; **23**: 28-31

12 **dos Santos JP**, Loureiro A, Cendoroglo Neto M, Pereira BJ. Impact of dialysis room and reuse strategies on the incidence of hepatitis C virus infection in haemodialysis units. *Nephrol Dial Transplant* 1996; **11**: 2017-2022

13 **Zampieron A**, Jayasekera H, Elseviers M, Lindley E, De Vos JY, Visser R, Harrington M. European study on epidemiology and the management of HCV in the haemodialysis population--Part 1: centre policy. *EDTNA ERCA J* 2004; **30**: 84-90

14 **Furusyo N**, Kubo N, Nakashima H, Kashiwagi K, Etoh Y, Hayashi J. Confirmation of nosocomial hepatitis C virus infection in a hemodialysis unit. *Infect Control Hosp Epidemiol* 2004; **25**: 584-590

15 **Izopet J**, Sandres-Sauné K, Kamar N, Salama G, Dubois M, Pasquier C, Rostaing L. Incidence of HCV infection in French hemodialysis units: a prospective study. *J Med Virol* 2005; **77**: 70-76

16 **Martin P**, Carter D, Fabrizi F, Dixit V, Conrad AJ, Artinian L, Peacock V, Han S, Wilkinson A, Lassman CR, Danovitch G. Histopathological features of hepatitis C in renal transplant candidates [see comment]. *Transplantation* 2000; **69**: 1479-1484

17 **Magnone M**, Holley JL, Shapiro R, Scantlebury V, McCauley J, Jordan M, Vivas C, Starzl T, Johnson JP. Interferon-alpha-induced acute renal allograft rejection. *Transplantation* 1995; **59**: 1068-1070

18 **Rostaing L**, Izopet J, Baron E, Duffaut M, Puel J, Durand D. Treatment of chronic hepatitis C with recombinant interferon alpha in kidney transplant recipients. *Transplantation* 1995; **59**: 1426-1431

19 **Kamar N**, Sandres-Saune K, Selves J, Ribes D, Cointault O, Durand D, Izopet J, Rostaing L. Long-term ribavirin therapy in hepatitis C virus-positive renal transplant patients: effects on renal function and liver histology. *Am J Kidney Dis* 2003; **42**: 184-192

20 **Kamar N**, Toupance O, Buchler M, Sandres-Saune K, Izopet J, Durand D, Rostaing L. Evidence that clearance of hepatitis C virus RNA after alpha-interferon therapy in dialysis patients is sustained after renal transplantation. *J Am Soc Nephrol* 2003; **14**: 2092-2098

21 **Tan AC**, Brouwer JT, Glue P, van Leusen R, Kauffmann RH, Schalm SW, de Vries RA, Vroom B. Safety of interferon and ribavirin therapy in haemodialysis patients with chronic hepatitis C: results of a pilot study. *Nephrol Dial Transplant* 2001; **16**: 193-195

22 **Sporea I**, Golea O, Ursu C, Totolici C, Popescu A, Sirli R et al. Effect of alpha 2b Interferon Treatment in Haemodialysis Patients with Chronic C hepatitis. *Rom J Gastroenterol* 2001; **4**: 285-288

23 **Bruchfeld A**, Ståhle L, Andersson J, Schvarcz R. Ribavirin treatment in dialysis patients with chronic hepatitis C virus infection--a pilot study. *J Viral Hepat* 2001; **8**: 287-292

24 **Rostaing L**. Treatment of hepatitis C virus infection after renal transplantation: new insights. *Nephrol Dial Transplant* 2000; **15** Suppl 8: 74-76

25 **Rostaing L**, Izopet J, Moussion F, Alric L, Verdier D, That HT, Duffaut M, Durand D, Puel J, Suc JM. HCV RNA clearance after treatment with interferon-alpha in chronic hemodialysis patients with or without coinfection by HGV/HGBV-C. *Nephrologie* 1997; **18**: 281-286

26 **Izopet J**, Rostaing L, Moussion F, Alric L, Dubois M, That HT, Payen JL, Duffaut M, Durand D, Suc JM, Puel J. [High rate of hepatitis C virus clearance in hemodialysis patients after interferon-alpha therapy]. *J Infect Dis* 1997; **176**: 1614-1617

27 **Huraib S**, Iqbal A, Tanimu D, Abdullah A. Sustained virological and histological response with pretransplant interferon therapy in renal transplant patients with chronic viral hepatitis C. *Am J Nephrol* 2001; **21**: 435-440

28 **Martin P**, Mitra S, Farrington K, Martin NE, Modi WN. Pegylated (40ku) Interferon alpha 2a (Pegasys) is unaffected by renal impairment (Abstract). *Hepatology* 2000; **32**: 842

29 **Russo MW**, Ghalib R, Sigal S, Joshi V. Randomized trial of pegylated interferon alpha-2b monotherapy in haemodialysis patients with chronic hepatitis C. *Nephrol Dial Transplant* 2006; **21**: 437-443

30 **Potthoff A**, Wiegand J, Lüth JB, Wedemeyer H, Manns MP, Tillmann HL. Superiority of standard interferon-alpha2b compared to pegylated interferon-alpha2b (12 kDa) in a hemodialysis patient with chronic hepatitis C? *Clin Nephrol* 2005; **63**: 232-235

31 **Mahmoud IM**, Sobh MA, El-Habashi AF, Sally ST, El-Baz M, El-Sawy E, Ghoneim MA. Interferon therapy in hemodialysis patients with chronic hepatitis C: study of tolerance, efficacy and post-transplantation course. *Nephron Clin Pract* 2005; **100**: c133-c139

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Circulating hTERT mRNA as a tumor marker in cholangiocarcinoma patients

Kawin Leelawat, Surang Leelawat, Thawee Ratanachu-Ek, Somboon Trubwongchareon, Jerasak Wannaprasert, Saad Tripongkaruna, Suchart Chantawibul, Panadda Tepaksorn

Kawin Leelawat, Thawee Ratanachu-Ek, Somboon Trubwongchareon, Jerasak Wannaprasert, Saad Tripongkaruna, Suchart Chantawibul, Department of Surgery, Rajavithi Hospital, Bangkok, 10400, Thailand
Kawin Leelawat, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand
Surang Leelawat, Faculty of Pharmacy, Rangsit University, Patumthani, 12000, Thailand
Panadda Tepaksorn, Medical Biotechnology Center, National Institute of Health, 13000, Thailand
Supported by Thailand Research Fund and Rajavithi Hospital Fund
Correspondence to: Kawin Leelawat MD, PhD, Department of Surgery, Rajavithi Hospital, Rajavithi Rd, Rajathevi, Bangkok, 10400, Thailand. kawin_leelawat@hotmail.com
Telephone: +66-2-3548080 Fax: +66-2-3548080
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Abstract

AIM: To investigate human telomerase reverse transcriptase (hTERT) mRNA in the serum of cholangiocarcinoma patients.

METHODS: The serum of thirty three cholangiocarcinoma patients, forty one benign biliary tract disease patients and ten healthy volunteers were collected and analyzed for the expression of hTERT mRNA by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). We then examined the correlation between values of serum hTERT mRNA and the pathological staging of cholangiocarcinoma.

RESULTS: hTERT mRNA was detected in 28 of 33 (84.85%) of serum obtained from cholangiocarcinoma patients and 9 of 41 (21.9%) of serum obtained from benign biliary tract disease patients. hTERT mRNA was not detected in any serum obtained from healthy volunteers. on the other hand the common tumor marker, CA19-9 was detected in 20 of 33 (60.6%) of serum obtained from cholangiocarcinoma patients and 8 of 41 (19.5%) of serum obtained from benign biliary tract disease patients. However, no correlation was found between the present of serum hTERT mRNA and tumor staging.

CONCLUSION: These results indicate that the detection of circulating hTERT mRNA was identified in almost all cholangiocarcinoma patients. It offers a novel tumor marker, which can be used as a complementary study for diagnosis of cholangiocarcinoma.

Key words: Cholangiocarcinoma; Telomerase; hTERT; Real-time reverse transcriptase-polymerase chain reaction

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INTRODUCTION

Cholangiocarcinoma is the cancer arising from cholangiocyte, the epithelial cells lining the intrahepatic and extrahepatic bile ducts. It is one of the most common liver cancers in the population of Northeast Thailand and responsible for approximately one in five cancer-related deaths among Thai patients[1]. Three-year survival rates of 40%-60% have been reported only in a few number of patients resected for cure[2]. Diagnosis of cholangiocarcinoma is often difficult. It requires multiple complementary studies including evaluation of clinical symptoms, imaging, and tumor markers. Tissue biopsy and cytology have poor sensitivity and are positive only in about 30% of cases of cholangiocarcinoma. Recently, the percentages of positive serum obtained from the common marker (CA19-9) are only less than 70%[3]. In addition, CA19-9 can be elevated in cholestasis in the absence of malignancy, and following liver injury. Thus, their accuracy for the diagnosis of cholangiocarcinoma is limited. It is necessary to find novel markers to use in diagnosis and treatment.

The human telomerase, which composed of two subunits including telomerase RNA template (hTR) and telomerase transcriptase protein (hTERT), functions as a reverse transcriptase enzyme in the process of telomere synthesis[4]. Telomerase activity was detected in 85%-100% of cancer patients whereas normal somatic cells have low or undetectable[4,5]. In addition, previous results demonstrated that circulating tumor-related RNA

including telomerase is frequently found in the plasma and serum of cancer patients[6-8].

Consequently, telomerase activity is possibly used as a common molecular tumor marker in the serum. Previous studies also found a good correlation between the telomerase activity and the expression of hTERT subunit. The aim of this study is to test the usefulness of hTERT mRNA detection in the serum of cholangiocarcinoma patients by using real-time reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Cell lines

The human cholangiocarcinoma cell line HuCCA1 (kindly provided by Prof. Sirisinha, Department of Microbiology, Mahidol University) and RMCCA1 (established from Department of Surgery, Rajavithi Hospital) were grown in Ham's F12 medium supplemented with 100 mL/L fetal bovine serum at 37℃ in a 5% (50 mL/L) CO_2 humidified atmosphere.

Patients and sample preparation

Thirty-nine informed and consenting patients undergoing surgery for cholangiocarcinoma at the Rajavithi Hospital, Thailand, between July 2003 and April 2006 were included in this study. Tumor samples were collected at the time of surgery and histopathologically characterized to confirm the diagnosis. Pathological data, including tumor staging was also collected. Fifty patients undergoing surgery for benign biliary tract disease were included in this study. Ten normal subjects were studied as negative controls.

Sample Collection

Blood was collected prior to surgery in plain tubes for serum sampling. After clotting, tubes were centrifuged at 1000 r/min for 15 min at room temperature, and serum was collected. This was followed by a second 15-min centrifugation at 1000 r/min to remove cellular debris. Serum samples were aliquoted and stored at -70℃ until use. Serum CA19-9 level was measured in Clinical Laboratory of Rajavithi Hospital. The cut-off level was 100 IU/mL.

RNA Extraction

RNA from cell lines and serum was extracted using a commercially available kit (High Pure RNA Kit; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Only fresh or once-freeze thawed serum was used.

Real time RT-PCR for hTERT mRNA

Quantitative detection of hTERT mRNA was performed with the TeloTAGGG hTERT Quantification Kit (Roche Diagnostics GmbH, Mannheim, Germany), using the LightCycler system (Roche Diagnostics, Mannheim, Germany) for real-time PCR according to the manufacturer's instructions. For the reaction mixtures, 2 μL of hTERT reaction mix, 0.1 μL of reverse transcriptase, 2 μL of hTERT or PBGD mix, 13.9 μL of H_2O and 2 μL of standard RNA template or RNA from serum samples was prepared. The reaction conditions were reverse transcription at 60℃ for 10 min, followed by initial denaturation at 95℃ for 30 s and 40 cycles of denaturation at 95℃ for 0.5 s, annealing at 60℃ for 10 s, and extension at 72℃ for 10 s, respectively. The standard curve was established by determination of the five standards hTERT mRNA provided by the kit. The samples were normalized on the basis of the content of PBGD. Serum samples with more than 150 copies of PBGD suggesting an appropriate quality of RNA were used for the analysis of telomerase. Serum samples in which hTERT mRNA were detected were assigned to the hTERT-positive group.

Table 1 The patient demographic data and blood chemistry data

	Benign biliary tract disease	Cholangio-carcinoma	Healthy volunteers
Sex (Male:Female)	21:20	19:14	6:4
Age (year), (median)	53.68 (22-82)	56.40 (35-85)	49.50 (26-60)
SGOT (IU/dL)	105.32 ± 52.26	112.52 ± 44.54	30.20 ± 12.20
SGPT (IU/dL)	96.04 ± 45.42	73.41 ± 44.30	28.50 ± 9.22
Total Bilirubin (mg/dL)[a]	3.8 ± 2.21	12.8 ± 5.24	1.05 ± 0.25
Alkaline Phosphatase (U/dL)[a]	309 ± 67.53	550 ± 24.44	98 ± 10.60

[a]$P < 0.005$.

Statistical analysis

Values were expressed as mean ± SD. Mean values were measured by the Student's *t* test. Correlations between serum hTERT mRNA and stage of cholangiocarcinoma were assessed using the chi-square test (χ^2). Sensitivity, specificity, positive predictive value, and negative predictive value were measured. $P < 0.05$ was considered as statistically significant.

RESULTS

Detection of hTERT mRNA in cholangiocarcinoma cells

The expression of hTERT mRNA in two cholangiocarcinoma cell lines (HuCCA1 and RMCCA1) was investigated. Quantitative real-time RT-PCR demonstrated definite expression of hTERT mRNA in both cholangiocarcinoma cell lines (Figure 1). This evidence confirmed the existence of telomerase activity in cholangiocarcinoma. Therefore, we attempted to detect the circulating hTERT mRNA in the serum of cholangiocarcinoma patients.

Detection of serum hTERT mRNA

Thirty-nine patients who were confirmed diagnosis as cholangiocarcinoma, fifty benign biliary tract disease patients and ten healthy volunteers were included in this study. Their serum was collected and extracted for total RNA. Only RNA samples, that could be detected by the expression of porphobilinogen deaminase (PBGD) as a housekeeping gene were included in this study. The thirty-three serum samples from cholangiocarcinoma patients, fortyone serum samples from benign biliary tract disease patients and ten serum samples from healthy volunteers detected for PBGD were as-

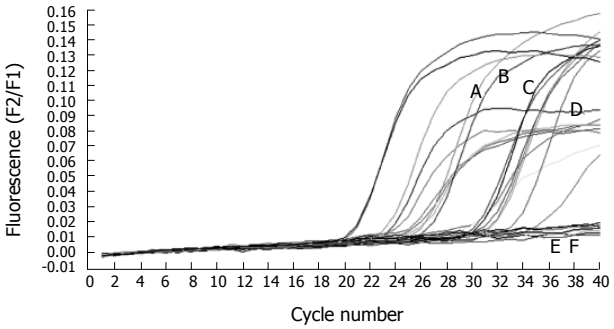

Figure 1 Continuously monitoring the development of signals in parallel standards and samples results in a series of amplification curves. The amplification of a 198 bp fragment of the generated hTERT cDNA was identified. (A: Signal from RMCCA1; B: Signal from HuCCA1; C, D: Signals from serum of cholangiocarcinoma patients; E, F: Signals from serum of benign biliary tract patients).

sayed for the expression of hTERT mRNA (Figure 1). The patients' demographic data was demonstrated in Table 1. There were no differences in sex, age, serum SGOT and serum SGPT between benign and cancer patients. However, total bilirubin and alkaline phosphatase were significantly high in cancer groups.

Serum hTERT mRNA was recognized in 28 of 33 cholangiocarcinoma patients (84.85%) and 9 of 41 benign biliary tract disease patients (21.9%). However, serum hTERT mRNA was not detected in any healthy volunteers. The efficiency of serum hTERT was compared with the serum CA19-9 as shown in Table 2. Serum hTERT was higher in sensitivity for detection of cholangiocarcinoma than serum CA19-9.

Detection of serum hTERT mRNA in relation to tumor stage

We also evaluated for the association between serum hTERT mRNA and the histopathological staging of cholangiocarcinoma in the surgical specimens resected from these patients. The result showed that serum hTERT mRNA could be detected in all stages of cholangiocarcinoma patients. However, it did not correlate with the staging of cholangiocarcinoma (Table 3).

Table 2 Comparative analysis of serum hTERT mRNA and serum CA19-9 in diagnosis of cholangiocarcinoma

	Serum hTERT (%)	Serum CA19-9 (%)
Sensitivity	84.85	60.6
Specificity	78.05	80.49
Positive predictive value	75.68	71.43
Negative predictive value	86.49	71.74
False Negative	13.51	28.26
False Positive	24.32	28.6

Table 3 Detection of serum hTERT mRNA in relation to tumor staging

Tumor stages	Serum hTERT mRNA positive	negative
Stage I	2	1
Stage II	17	3
Stage III	9	1

DISCUSSION

Eukaryotic chromosomal ends consist of repeating DNA sequences (TTAAGG) termed telomeres. An enzyme that adds telomeric repeats onto chromosomal ends is telomerase. This enzyme is composed of two subunits; hTR and hTERT[4]. Accordingly, both hTR and hTERT are necessary for telomerase activity, yet the catalytic activity of the enzyme is generally regulated through the presence and activity of hTERT. Therefore, the detection of hTERT mRNA is a guarantee for the present of telomerase activity[8]. In most somatic cells in which telomerase activity is undetectable, telomeric sequences are lost with each cell division because of the end-replication problem. Unlike healthy cells, most malignant human cells are capable of escaping senescence and sustaining infinite proliferation through the activation of telomerase to stabilize their telomere length[4,8-12].

Traditionally, telomerase activity has been assessed based on a biochemical primer extension assay, the inefficiency and low sensitivity of which, together with the low amounts of telomerase activity, greatly limit the application of the assay in primary human tumors[9]. Therefore, the detection of hTERT by real-time RT-PCR was used in this study. This assay determined the expression of telomerase by measuring the amount of the mRNA encoding its catalytic subunit hTERT. The relative telomerase expression levels were determined by comparing them to the expression levels of the housekeeping gene porphobilinogen deaminase (PBGD). hTERT-encoding mRNA from the serum was reverse transcribed and 198 bp fragments of the generated cDNA was amplified with specific primers in a one step RT-PCR reaction. The amplicon was detected by fluorescent emission using a specific pair of hybridization probes. In a separate RT-PCR, mRNA encoding for porphobilinogen deaminase (PBGD) was processed. The reaction product served as both a control for RT-PCR performance and as a reference for relative quantification.

Our study showed that hTERT mRNA was detected in almost all of the cholangiocarcinoma patients (84.85% of cases). However, in benign biliary tract disease patients, hTERT mRNA was also detectable (21.9% of cases). According to the previous report, telomerase activity has been reported in normal lymphocytes[8-12]. This result suggested the contamination of lymphocytes in the serum specimens. Comparison with the common tumor marker, CA19-9 was detected in only 60.60% of cases. This data suggested that hTERT mRNA should be a candidate tumor marker in cancer patients.

hTERT mRNA is not specifically detected in serum of cholangiocarcinoma patients but it was also significantly found in several types of cancers such as breast cancer, malignant melanoma and thyroid cancer[8]. Certainly, we have detected hTERT mRNA in the serum of five patients with hepatocellular carcinoma and three patients with pancreatic cancers (data not shown). This indicates that the

detection of circulating extracellular tumor-derived mRNA is not confined to any one cancer type, but may actually be a relatively ubiquitous finding across a broad range of cancers. However, no relationship was found between the expression of hTERT mRNA and clinicopathological findings. According with previous study, the presence of hTERT was unrelated to tumor size, tumor grade or the presence of nodal metastasis[12]. These results suggest that the detection circulating hTERT mRNA does not predict prognosis in cholangiocarcinoma.

In conclusion, hTERT might serve as a tumor marker, which early identified circulating specific RNA originating from tumor cells. However, further examinations using more cholangiocarcinoma cases are necessary to evaluate the usefulness of this marker.

REFERENCES

1 **Sriplung H**, Sontipong S, Martin N, Wiangnon S, Vootiprux V, Cheirsilpa A, Kanchanabat C, Khuhaprema T. Cancer incidence in Thailand, 1995-1997. *Asian Pac J Cancer Prev* 2005; **6**: 276-281

2 **Chamberlain RS**, Blumgart LH. Hilar cholangiocarcinoma: a review and commentary. *Ann Surg Oncol* 2000; **7**: 55-66

3 **Olnes MJ**, Erlich R. A review and update on cholangiocarcinoma. *Oncology* 2004; **66**: 167-179

4 **Bachand F**, Triki I, Autexier C. Human telomerase RNA-protein interactions. *Nucleic Acids Res* 2001; **29**: 3385-3393

5 **Yan P**, Coindre JM, Benhattar J, Bosman FT, Guillou L. Telomerase activity and human telomerase reverse transcriptase mRNA expression in soft tissue tumors: correlation with grade, histology, and proliferative activity. *Cancer Res* 1999; **59**: 3166-3170

6 **Johnson PJ**, Lo YM. Plasma nucleic acids in the diagnosis and management of malignant disease. *Clin Chem* 2002; **48**: 1186-1193

7 **Fleischhacker M**, Beinert T, Ermitsch M, Seferi D, Possinger K, Engelmann C, Jandrig B. Detection of amplifiable messenger RNA in the serum of patients with lung cancer. *Ann N Y Acad Sci* 2001; **945**: 179-188

8 **Novakovic S**, Hocevar M, Zgajnar J, Besic N, Stegel V. Detection of telomerase RNA in the plasma of patients with breast cancer, malignant melanoma or thyroid cancer. *Oncol Rep* 2004; **11**: 245-252

9 **Mabruk MJ**, O'Flatharta C. Telomerase: is it the future diagnostic and prognostic tool in human cancer? *Expert Rev Mol Diagn* 2005; **5**: 907-916

10 **Chen XQ**, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, Schaeffer P, Mulcahy HE, Meyer P, Stroun M, Anker P. Telomerase RNA as a detection marker in the serum of breast cancer patients. *Clin Cancer Res* 2000; **6**: 3823-3826

11 **Blasco MA**. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* 2005; **6**: 611-622

12 **Li YR**, Wu JM, Wang L, Huang X, Shi J, Hu LH. Human telomerase reverse transcriptase expression and its clinical significance in laryngeal squamous cell carcinoma. *Acta Otolaryngol* 2005; **125**: 409-414

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Results of gastroscope bacterial decontamination by enzymatic detergent compared to chlorhexidine

Rungsun Rerknimitr, Sorapat Eakthunyasakul, Pongpan Nunthapisud, Pradermchai Kongkam

Rungsun Rerknimitr, Sorapat Eakthunyasakul, Pradermchai Kongkam, Gastroenterology Unit, Department of Internal Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
Pongpan Nunthapisud, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
Correspondence to: Rungsun Rerknimitr, MD, Gastroenterology Unit, Department of Internal Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. rungsun@pol.net
Telephone: +66-2-2564265 Fax: +66-2-2527839
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Abstract

AIM: To compare the efficacy of enzymatic detergent with chlorhexidine for gastroscope bacterial decontamination.

METHODS: A prospective randomized controlled study was undertaken to evaluate the ability of these 2 agents to achieve high level disinfection in a gastroscope. A total of 260 samples were collected from 5 different gastroscopes. Manual cleaning was done for 10 min with these 2 agents separately (n = 130 each). Then all specimens underwent 2% glutaraldehyde soaking for 20 min. After 70% alcohol was rinsed, sterile normal saline was flushed into each gastroscope channel and 40 mL of sample was collected. The sample was sent for aerobic bacterial culture after membrane was filtered. A colony count greater than 200 cfu/mL was considered significant.

RESULTS: The positive culture rate was 4.6% in the enzymatic detergent arm and 3.1% in the chlorhexidine arm. Pseudomonas species were the main organism detected from both groups (60%). Multiple organisms were found from 4 specimens (enzymatic detergent arm = 1, chlorhexidine arm = 3).

CONCLUSION: The contamination rate of both types of cleaning solution is equivalent.

Key words: Enzymatic detergent; Gastroscope; Bacterial decontamination

Rerknimitr R, Eakthunyasakul S, Nunthapisud P, Kongkam P. Results of gastroscope bacterial decontamination by enzymatic detergent compared to chlorhexidine. *World J Gastroenterol* 2006; 12(26): 4199-4202

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INTRODUCTION

The endoscope is a complex, reusable device that requires reprocessing before being used in subsequent patients. Generally, a high-level of disinfection is required for reprocessing endoscopes[1-2]. To date, all published incidents of pathogen transmission related to gastrointestinal (GI) endoscopy are associated with failure to follow established cleaning and disinfection/sterilization guidelines or with the use of defective equipments[3-5].

Guidelines for reprocessing flexible gastrointestinal endoscopes have been recommended by several professional organizations[6-10]. However, different professional organizations do not have similar recommended practices[6-9]. Cleaning solutions are one of the different factors. In USA, multi-society guidelines for reprocessing flexible gastrointestinal endoscopes recommend to use enzymatic detergent as an initial endoscopic cleaning agent[1]. However, different countries select different agents for this purpose. Chlorhexidine is one of the popular solutions that have been accepted for endoscope cleaning in Thailand. Unfortunately, there are some reports on bacterial transmission from this standard endoscope reprocessing practice[7]. Bacterial biofilm is known to interfere with the cleaning efficacy of chlorhexidine. Biofilms consist of colonies of organisms forming structures to maximize growth potential. The ability of bacteria to form biofilms is an important factor in the pathogenesis of endoscopy-related infections, particularly as biofilms interfere with disinfection. Strategies aimed at decreasing biofilm formation and viability play an important role in endoscope disinfection because biofilms adhere to the internal channels of endoscopes[4,7].

Recently, many professional organizations have accepted enzymatic detergent for endoscope cleaning[1,7,9,11]. However, there is no randomized controlled study to demonstrate the efficacy of this agent for scope cleaning over chlorhexidine. Hence, the aim of this study was to evaluate the cleaning ability of these 2 agents combined with a standard disinfectant like glutaraldehyde to achieve high level disinfection for gastroscope cleaning.

MATERIALS AND METHODS

A prospective randomized controlled study was undertaken to evaluate the cleaning capacity of gastroscope reprocessing by 3E-ZYME (Medisafe UK Limited, Hartfordshire, UK) and hexene (Osoth Inter Laboratoreis, Chonburi, Thailand). All specimens were collected at the Gastroenterology Unit, King Chulalongkorn Memorial Hospital between July 2004 and October 2004. A total of 260 samples were collected from 5 different gastroscopes. These samples were divided into two groups by stratified randomization and block of 4. Group 1 (n = 130) received enzymatic detergent during endoscope cleaning, and group 2 (n = 130) received chlorhexidine detergent during endoscope cleaning.

The 3E-ZYME is a non-foaming, triple enzymatic detergent and designed for use in endoscope processing. It has a neutral pH formulation and is safe for instruments when used as directed. The directions indicate that 3E-ZYME should be diluted 3-7 milliliters (mL) to every liter (L) of warm (40℃-60℃) water and that the devices should be immersed for 1 min. In the other group, hexene was used as the conventional cleaning detergent. Hexene is an aqueous solution of 4% (weight/volume) chlorhexidine gluconate. In the present study, hexene was diluted from 25 mL to 5 L with filtered water, and the endoscopes were also immersed for 10 min.

Gastroscope reprocessing was performed in accordance with recognized standards for infection control and endoscope reprocessing. All personnel were well trained to comply with the protocol. The protocol for gastroscope reprocessing in the present study is shown in Table 1. Endoscope was randomly selected to be cleaned by one of the two cleaning agents. After gastroscope reprocessing was completely performed, a sample was collected by the flush method (injecting sterile water from the top of accessory channel of the endoscope and subsequently, the sample was collected from the distal tip of the endoscope). All samples were sent for aerobic bacterial cultures using a membrane filtering. Anaerobic bacterial, fungal and viral cultures were not performed due to insufficient information regarding the effect of bacterial biofilm over these organisms.

For quantitative culture, membrane filter method was performed in this study (limit of detection, 1 cfu/specimen). All inoculated plates were incubated aerobically at 37℃ for 24-48 h before the number of colonies was counted. Culture results were variably reported as colony counts per milliliter. A colony count greater than 200 cfu/mL was considered significant.

Chulalongkorn University Institutional Board Review approved and supported all ethical issues related to this study. Descriptive statistics were expressed as n (%). Statistical analysis was performed by chi-square or Fisher's exact test. $P < 0.05$ was considered statistically significant. Data were analyzed with the Statistic of Package for Social Sciences (SPSS 11.5) program (Chicago, IL, USA).

RESULTS

All the five gastroscopes were equally distributed in the 2 groups (Table 2). The rates of bacterial contamination (> 200 cfu/mL) in both groups are shown in Table 3. The positive culture rate was 4.6% from the enzymatic detergent group and 3.1% from the chlorhexidine group. This was not statistically significant (P = 0.747).

Overall, the rate of bacterial contamination was 3.85% (10/260 samples). The incidences and types of organisms during study period are shown in Table 4. The most common organism was Pseudomonas (60%) in group 1 (n = 4, 3.1%) and group 2 (n = 5, 3.8%) (Table 3). Other organisms included Klebseilla species (13.33%), Enterobacter species (6.66%), Acinetobacter baumannii

Table 1 Steps for gastroscope reprocessing in the present study

Gastroscope reprocessing
Cleaning After completion of the cleaning procedure, the inserted tube was wiped with a wet cloth and soaked in detergent solution (chlorhexidine or 3E-ZYME). Detergent solution was suctioned through the biopsy channel until the solution was visibly clean.
While the scope was submerged, mechanical cleaning was performed by washing all debris from the exterior. All removable parts were separately cleaned. A soft cleaning brush was used to clean all accessible channels. Manual cleansing was done for 10 min.
The scope was removed from the detergent solution and then submerged in 5 L of filtered water. An all-channel irrigator was used to flush water through it.
Leak testing of the scope was performed.
Disinfection After manual cleaning, the gastroscope underwent high-level disinfection in a container using 2% glutaraldehyde with a 20-min soak time.
The scope was removed from 2% glutaraldehyde and then submerged in 5 L of filtered water. An all-channel irrigator was used to flush water through it.
Rinsing and Drying The suction/biopsy channel was rinsed with 70% alcohol 20 mL and dried for 5 min.
The suction/biopsy channel was sampled using the flush method.

Table 2 Characteristics of endoscopes in both groups

	Enzymatic detergent	Chlorhexidine
Specimen (n)	130	130
Endoscopes		
Olympus GIF-V	30	30
Olympus GIF-IT 140	30	30
Pentax 2970 K	35	35
Pentax 2930 K	22	22
Pentax 3830 TK	13	13

Table 3 Results of bacterial contamination after gastroscope reprocessing in both groups

	Enzymatic detergent (n = 130)	Chlorhexidine (n = 130)	P
Type of endoscope (Olympus:Pentax)	60:70	60:70	
Positive culture (> 200 cfu/mL)	6 (4.6%)	4 (3.1%)	0.747[a]
Single organism	5 (3.8%)	1 (0.8%)	0.213[b]
Mixed organism	1 (0.8%)	3 (2.3%)	0.622[b]
Pseudomonas aeruginosa	4 (3.1%)	5 (3.8%)	1.000[b]
Non Pseudomonas spp.	3 (2.3%)	3 (2.3%)	1.000[b]

a: chi square test; b: Fisher's exact test.

(6.66%), Staphylococcus coagulase negative (6.66%) and Staphylococcus aureus (6.66%).

DISCUSSION

Ensuring safety in patients undergoing endoscopy, proper endoscope reprocessing is required. According to Spaulding classification of disinfection of medical and surgical instruments, flexible GI endoscope reprocessing is categorized as semicritical level since endoscopy has no involvement with tissue penetration[12].The reprocessing of endoscopes is susceptible to multiple errors, as it is a multi-step process relying on both human and material for reprocessing. The reprocessing involves meticulous manual cleaning and rinsing. This step is followed by high-level disinfection with liquid chemical germicide. Chlorhexidine is commonly used to decontaminate an endoscope prior to high level disinfection. However, recent reports from the Center of Disease Control and Prevention suggested that a significant number of infections are transmitted during endoscopic procedures after reprocessing these scopes under both manual and automated cleanings[7]. Detailed analysis of these cases has identified either a breakdown in the cleaning process or a damage by equipment as the causative factor[6,13]. It is possible that bacterial biofilm contributes to the failure of adequate endoscope reprocessing in certain instances. Vickery *et al*[14] showed that bacterial biofilm is an important factor in endoscope contamination, and that routine cleaning procedures do not remove biofilm reliably from endoscope channels. Generally, biofilm consisting of bacteria enclosed in a matrix of exopolysaccharide (EPS) can form on many medical devices such as catheters and endoscopes. Chemical cleaning methods by agents like chlorhexidine are often ineffective because biofilm has a strong resistance to these biocides. Biofilm removal by physical methods such as ultrasound and mechanical cleaning is reasonably effective but it is difficult to supervise in practice.

To solve this problem, agents that can be used to remove the bacterial biofilm during the process of endoscope cleaning are desirable. The efficacy of enzymatic cleaning agents to reduce the bacterial load and biofilm in laboratory setting has been studied recently[15]. In addition, the ASGE and the SHEA have recently endorsed enzymatic detergents in reprocessing endoscopes and reusable accessories[1].

Enzymatic detergents generally containing various combinations of protease, lipase and amylase, require a minimum contact time to enable them to adequately remove the bacterial biofilm[16]. To date, there has been no report on the bacterial decontamination rate of these enzymatic detergents for endoscope reprocessing.

The bacterial concentration cultured from an endoscope after decontamination is an important factor in determining the risk of transmission from an endoscope to a patient. At present there is no standard bacterial concentration above which the endoscope is considered contaminated. We used the AAMI[17,18] guidelines established for hemodialysis water, < 200 cfu/mL.

In our series, the overall rate of bacterial contamination above the cut off level from enzymatic detergent and chlorhexidine was very low (3.85%). This is significantly different from previous studies that mainly used non-enzymatic cleaning agents which demonstrated a contamination rate as high as 24%[1,5]. The majority of bacteria identified in this study were Gram-negative bacilli. Pseudomonas aeruginosa was the most common species. This is similar to other published series[19,20]. Though we did not observe any adverse clinical outcomes in the patients exposed to the contaminated endoscopes, the primary goals of this study were not to address this question.

Table 4 Incidence and types of organisms during study period

Type of organism	Enzymatic detergent (samples, *n*)	Chlorhexidine (samples, *n*)	Total, *n* (%)
Pseudomonas aeruginosa	4	5	9 (60)
Klebsiella species	1	1	2 (13.3)
Enterobacter species	1	0	1 (6.7)
Acinetobacter baumanii	0	1	1 (6.7)
Staphylococcus coagulase negative	1	0	1 (6.7)
Staphylococcus aureus	0	1	1 (6.7)
Total	7	8	15 (100)

A group from Walter Reed Army Medical Center reported that their surveillance of bacterial culture result from GI endoscopes is as high as 14.5% and that more than half of positive cultures are obtained from therapeutic scopes that were used during emergency procedure, which might be attributed to faulty mechanical cleaning by non-nursing personnel after emergent procedures[5]. Furthermore, adherence to the standard guideline for endoscope reprocessing can result in a low rate of disease transmission[1].

In conclusion, 4% chlorhexidine is not worse than enzymatic detergent for endoscope decontamination. Both of them have a very low rate of significant positive bacterial cultures. Further investigations on the effectiveness of the enzymatic agent on decontamination of other organisms apart from aerobic bacteria are required.

REFERENCES

1 Multi-society guideline for reprocessing flexible gastrointestinal endoscopes. *Gastrointest Endosc* 2003; **58**: 1-8
2 **Moses FM**, Lee J. Surveillance cultures to monitor quality of gastrointestinal endoscope reprocessing. *Am J Gastroenterol* 2003; **98**: 77-81
3 **Spach DH**, Silverstein FE, Stamm WE. Transmission of infection by gastrointestinal endoscopy and bronchoscopy. *Ann Intern Med* 1993; **118**: 117-128
4 **Nelson DB**, Barkun AN, Block KP, Burdick JS, Ginsberg GG, Greenwald DA, Kelsey PB, Nakao NL, Slivka A, Smith P, Vakil N. Technology status evaluation report. Transmission of infection by gastrointestinal endoscopy. May 2001. *Gastrointest Endosc* 2001; **54**: 824-828
5 **Donlan RM**. Biofilms: microbial life on surfaces. *Emerg Infect Dis* 2002; **8**: 881-890
6 **Alvarado CJ**, Reichelderfer M. APIC guideline for infection prevention and control in flexible endoscopy. Association for Professionals in Infection Control. *Am J Infect Control* 2000; **28**: 138-155
7 Recommended practices for cleaning and processing endoscopes

and endoscope accessories. *AORN J* 2003; **77**: 434-438, 441-442
8 **Standards of infection control in reprocessing of flexible gastrointestinal endoscopes**. *Gastroenterol Nurs* 2000; **23**: 172-179
9 Guidelines on cleaning and disinfection in GI endoscopy. Update 1999. The European Society of Gastrointestinal Endoscopy. *Endoscopy* 2000; **32**: 77-80
10 **Cleaning and disinfection of equipment for gastrointestinal endoscopy**. Report of a Working Party of the British Society of Gastroenterology Endoscopy Committee. *Gut* 1998; **42**: 585-593
11 **Reprocessing of flexible gastrointestinal endoscopes**. American Society for Gastrointestinal Endoscopy. *Gastrointest Endosc* 1996; **43**: 540-545
12 **Spaulding EH**. Chemical disinfection of medical and surgical materials. In: Lawrence CA, Black SS, eds. Disinfection, sterilization and preservation. Philadelphia: Lea & Febiger, 1968: 517-531
13 **Ahuja V**, Tandon RK. Survey of gastrointestinal endoscope disinfection and accessory reprocessing practices in the Asia-Pacific region. *J Gastroenterol Hepatol* 2000; **15** Suppl: G78-G81
14 **Vickery K**, Pajkos A, Cossart Y. Removal of biofilm from endoscopes: evaluation of detergent efficiency. *Am J Infect Control* 2004; **32**: 170-176
15 **Augustin M**, Ali-Vehmas T, Atroshi F. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *J Pharm Pharm Sci* 2004; **7**: 55-64
16 **Ayliffe G**. Decontamination of minimally invasive surgical endoscopes and accessories. *J Hosp Infect* 2000; **45**: 263-277
17 **Sehulster L**, Chinn RY. Guidelines for environmental infection control in health-care facilities. Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC). *MMWR Recomm Rep* 2003; **52**: 1-42
18 **Bond WW**, Hedrick ER. Microbial culturing of environment and medical device surfaces. In: Isenberg HD, Gilchrist MJR, eds. Clinical microbiology procedures handbook. Washington (DC): American Society for Microbiology, 1992: 11
19 **Chu NS**, McAlister D, Antonoplos PA. Natural bioburden levels detected on flexible gastrointestinal endoscopes after clinical use and manual cleaning. *Gastrointest Endosc* 1998; **48**: 137-142
20 **Foliente RL**, Kovacs BJ, Aprecio RM, Bains HJ, Kettering JD, Chen YK. Efficacy of high-level disinfectants for reprocessing GI endoscopes in simulated-use testing. *Gastrointest Endosc* 2001; **53**: 456-462

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Biochemical response to lamivudine treatment in HBeAg negative chronic hepatitis B patients in Iran

Amir Houshang Mohammad Alizadeh, Mitra Ranjbar, Babak Karimi, Saeed Hatami

Amir Houshang Mohammad Alizadeh, Research Center for Gastroenterology and Liver Disease, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
Mitra Ranjbar, Infectious Disease Specialist, Hamadan University of Medical Sciences and Health Services, Iran
Babak Karimi, Saeed Hatami, General Practitioner, Hamadan University of Medical Sciences and Health Services, Iran
Correspondence to: Amir Houshang Mohammad Alizadeh, Research Center for Gastroenterology and Liver Disease, Shaheed Beheshti University of Medical Sciences, 7th floor, Taleghani Hospital, Yaman Str., Evin, Tehran 19857, Iran. article@rcgld.org
Telephone: +98-21-2418871 Fax: +98-21-2402639
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Abstract

AIM: To study the effect of a one-year lamivudine regimen in patients with chronic hepatitis B.

METHODS: Medical records of HBeAg negative hepatitis B patients who attended a hepatitis clinic in Tehran between March 2002-March 2004 were evaluated. The patients received 100 mg lamivudine tablets once daily for at least 12 mo. Liver enzymes and complete blood count were checked at baseline and the end of treatment (12th mo) and 6 mo after discontinuation of treatment.

RESULTS: Of all patients, 24 were excluded. Of 71 patients left, 58 (81.7%) were men. Mean age of the patients was 38 ± 14 years. Mean level of ALT in serum was 1437 ± 205 nkat/L at baseline with a significant reduction at the end of treatment to a mean level of 723 ± 92 nkat/L (P = 0.002). In 38 patients (53.5%), the ALT level was normal after one-year treatment. Five patients (7.3%) relapsed (biochemically) within 6 mo after discontinuing lamivudine therapy (the patients with good end of treatment response). Mean level of AST in serum was 1060 ± 105 nkat/L at baseline which decreased significantly to 652 ± 75 nkat/L at the end of treatment (P = 0.002).

CONCLUSION: Over half (53.5%) of chronic hepatitis B patients with HBeAg negative have normal liver enzyme level at 12-mo lamivudine therapy.

Key words: Chronic hepatitis B; Lamivudine; HBeAg

Mohammad Alizadeh AH, Ranjbar M, Karimi B, Hatami S. Biochemical response to lamivudine treatment in HBeAg negative chronic hepatitis B patients in Iran. *World J Gastroenterol* 2006; 12(26): 4203-4205

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INTRODUCTION

Chronic hepatitis B is a pandemic disease which has been under constant focus because it has long-term serious complications such as hepatic cirrhosis and hepatocellular carcinoma, while it is a preventable condition. About 350 million people have the condition all around the world with 200 000 patients living in Iran[1]. Length of drug therapy, frequency of recurrences after primary remission, cost of drug regimen and resistance to treatment have led investigators to look for the best and most cost-effective therapeutic regimens for chronic hepatitis B in numerous studies throughout the world. A study in Canada estimated the annual cost of medical care for a patient with chronic hepatitis as high as $2191 while a cirrhotic patient's medical care costs summed up to an estimated $11 228 each year[2].

The objectives of treatment in chronic hepatitis B patients are suppression of viral replication as well as reduction in hepatic inflammation. Lamivudine is a nucleoside analogue which prevents the viral DNA synthesis. Markers such as HBeAg, liver enzymes especially ALT and HBV DNA are used for monitoring patients' response to treatment. Many studies assessed the effectiveness of a number of different therapeutic regimens in different community contexts[3,4]. In the present study, we investigated the effect of a one-year lamivudine regimen in HBeAg negative patients with chronic hepatitis B who attended a hepatitis clinic in Tehran.

MATERIALS AND METHODS

Patients

Ninety-five medical records of HBeAg negative hepatitis B patients who were registered in a hepatitis clinic in Tehran during March 2002 to March 2004 were evaluated; these patients had positive HBsAg, positive HBeAb, negative HBeAg and sustained or intermittent increased ALT for more than 6 mo. Serum HBV DNA loads were measured by quantitative real-time PCR with primers that amplify

Table 1 Frequency of response and serum liver enzymes during 12-mo treatment of HBeAg negative patients with lamivudine (n = 71)

	Baseline	3 mo		6 mo		12 mo	
		Mean	Frequency of response	Mean	Frequency of response	Mean	Frequency of response
ALT (nkat/L)	1437 ± 205	1060 ± 122	65 (91.5%)	813 ± 160	61 (85.9%)	723 ± 92	38 (53.5%)
AST (nkat/L)	1060 ± 105	960 ± 77	68 (95.7%)	770 ± 88	63 (88.7%)	652 ± 75	36 (50.1%)

P = 0.002 for ALT between baseline and 12 mo treatment levels; P = 0.002 for AST between baseline and 12 mo treatment levels.

a highly conserved region of the surface gene[5] and with a detection system that employed molecular beacon technology[6]. The assay was calibrated using international HBV standards and a plasmid control containing a full-length cloned copy of HBV ayw. The assay has a 7 log10 linear response, with a lower limit of detection of 2×10^5 copies/L and an intraassay coefficient of variability of 15%. All patients with a serum ALT level of more than 2 times of upper normal limit, serum HBV DNA more than 10^5 copies/mL with histopathologic features in liver biopsy consistent with chronic hepatitis ($\geqslant$ F2) in the Knodell score, [also known as the histologic activity index (HAI)], were included. Those who had evidence of other liver diseases such as hepatitis C, autoimmune liver diseases, non alcoholic fatty liver disease (NAFLD) and non alcoholic steatohepatitis (NASH) in sonography and liver biopsy, and biliary tract diseases, thyroid diseases were excluded. Also, all cases of non-compliance with therapy were excluded.

Methods

Lamivudine was prescribed at a dosage of 100 mg daily. All patients had used the drug for at least 12 mo. Liver enzymes (AST, ALT), complete blood cell counts were checked and recorded at baseline, mo 3 and 6 of treatment, the end of one year treatment and 6 mo after discontinuation of treatment. Forty-two patients had detected serum HBV DNA loads at the end of treatment for their own interest in evaluation of some virological responses.

Statistical analysis

The data were analyzed by SPSS and paired t test was used to compare the variables before and after treatment. $P < 0.05$ was taken as significant.

RESULTS

Of all medical records, 24 were withdrawn from the study: 16 patients did not take the prescribed drug correctly, and 8 patients had a follow-up period of less than 6 mo. Of 71 patients left, 58 were men. Mean age of the patients was 38 ± 14 years. The youngest patient was 12 years old while the oldest was 71. Mean weight of patients was 68 ± 14 kg. The lowest weight was 38 kg while the highest was 115 kg. Biochemical markers are as follows: Mean level of hemoglobin was 144 ± 17 g/L with lowest value of 109 g/L and highest value of 175 g/L. Mean white blood cells count was (6.39 ± 2.2) × 10^9/L with lowest count of 3.1 × 10^9/L and highest count of 11.6 × 10^9/L. Mean prothrombin time was 14.3 ± 4.5 s at baseline with shortest time of 11 s and longest time of 46 s. Mean platelet count was (199.9 ± 9.03) × 10^9/L. Of all patients, 20 (28.2%) were diagnosed by clinical manifestation while a major proportion of patients (32%-45.0%) were diagnosed incidentally by routine examination on the blood they donated. Only 7 patients were diagnosed through screening and checkups.

Table 2 Liver serum markers after 3 and 6 mo of termination of one-year regimen in patients who responded to treatment (n = 38)

	12 mo	After 3 mo		After 6 mo	
	Mean	Mean	Frequency of normal level	Mean	Frequency of normal level
ALT (nkat/L)	538 ± 90	688 ± 157	28 (73.7%)	902 ± 238	25 (65.8%)
AST (nkat/L)	447 ± 122	677 ± 143	32 (84.2%)	803 ± 210	28 (73.7%)

$P < 0.04$ between 12 mo of and after 6 mo cessation of treatment for ALT, $P < 0.03$ between 12 mo of and after 6 mo cessation of treatment for AST.

In terms of probable routes of transmission, 43.7% had a history of dental procedure, 15.5% had a tattoo and 12.7% underwent a surgical procedure. Of all patients, 44 (62%) had no family history of hepatic disease. Those who had a positive family history had a sibling with the condition, while 4 mothers and 8 fathers had the condition.

A biopsy was taken from 37 patients before the treatment. The biopsy samples were reported based on Knodell score. Of these samples, 73% had a score of less than 6. The liver enzyme levels before, during and after treatment with lamivudine were compared. As shown in Table 1, mean level of ALT in serum was 1437 ± 205 nkat/L at baseline with a significant reduction after treatment to a mean level of 723 ± 92 nkat/L (P = 0.002). In 53.5% of patients the ALT level was normal after one-year treatment. Mean level of AST in serum was 1060 ± 105 nkat/L at baseline which decreased significantly to 652 ± 75 nkat/L after treatment (P = 0.002). Of 38 (53.5%) patients who responded to treatment, 28 (73.7%) and 25 (65.8%) patients had normal ALT levels after 3 and 6 mo of termination of one-year regimen, respectively. There was no significant elevation of ALT and AST after 3 mo of cessation of treatment compared to 12 mo of treatment, while there was a significant difference between ALT and AST level at 6 mo after termination of and 12 mo of treatment ($P < 0.04$ and $P < 0.03$, respectively, Table 2). At the end of treatment, HBV DNAs were measured in 42 cases and was negative in 24 cases.

Mild adverse events were seen in 12 patients including fatigue in five patients, headache in three, myalgia in two, decreased appetite in two, but there were no reports of therapy discontinuation.

DISCUSSION

The major proportion of the patients were men and the age distribution of sample can be regarded as an adult population. The probable routes of transmission in order of frequency were a history of dental procedure, having a tattoo and a history of surgical procedure, which is consistent with other studies[7,8]. However, to confirm these results, studies to evaluate the precise role of these risk factors in the transmission of hepatitis B are needed. It is clear that the risk of transmission of hepatitis *via* these procedures has not been evaluated completely in our study. The most frequent event leading to diagnosis was blood donation, which is consistent with other studies[9]. The most significant factor under focus in our study was the changes in serum levels of liver enzyme especially ALT after treatment by lamivudine. As presented earlier the reduction in serum level of ALK, AST, and ALT were significant at 12 mo of therapy.

In a 3-year retrospective study in the United States, the therapeutic response markers of 119 patients were analyzed. The results showed that 61% of patients had a normal ALT level after treatment. The ALT serum level at baseline was not a significant predictor for response to therapy by lamivudine[10]. Similar results were reported by a Chinese study on 129 patients with chronic hepatitis. In the current study 60.3% of patients completing a one-year lamivudine treatment had a normal ALT level while the ALT level was normal in only 24.5% of controls at 12 mo. The difference was significant[11]. A recent study in Pakistan showed that a course of one-year lamivudine treatment was associated with significant reduction in ALT serum level, the treatment was more effective in patients with HBeAg at baseline[12]. In another study in Austria, 72% of the intervention group had normal ALT level while 29% of controls had normal ALT level[13]. Most studies of one-year lamivudine treatment for patients with chronic hepatitis B showed a reduction of liver enzyme to normal level at 12 mo[14-18]. In our study, 53.5% of patients had normal liver enzyme level at 12 mo of lamivudine therapy, which is slightly lower than that of other studies.

In patients whose enzyme level was not normal after lamivudine treatment (50% of patients), it is likely that a resistant mutant is present which warrants further study for confirmation.

REFERENCES

1 **Malekzadeh R,** Khatibian M, Rezvan H. Viral hepatitis in the world and in Iran: epidemiology, diagnosis, and follow up. *Scien J Med Council* 1997; **15**: 183-200

2 **Gagnon YM,** Levy AR, Iloeje UH, Briggs AH. Treatment costs in Canada of health conditions resulting from chronic hepatitis B infection. *J Clin Gastroenterol* 2004; **38**: S179-S186

3 **Dybowska D**, Pilarczyk M. [Treatment of chroni hepatitis B]. *Przegl Epidemiol* 2004; **58** Suppl 1: 139-143

4 **Figlerowicz M,** Kowala-Piaskowska A, Filipowicz M, Bujnowska A, Mozer-Lisewska I, Służewski W. Efficacy of lamivudine in the treatment of children with chronic hepatitis B. *Hepatol Res* 2005; 31: 217-222

5 **Abe A,** Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, Tanaka S, Yoshiba M, Kohara M. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol* 1999; **37**: 2899-2903

6 **Vet JA,** Majithia AR, Marras SA, Tyagi S, Dube S, Poiesz BJ, Kramer FR. Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc Natl Acad Sci USA* 1999; **96**: 6394-6399

7 **Mohammadalizadeh AH**, Ranjbar M, Hatami S. Risk factor for HBsAg positive state in Hamedan blood donors. *Iranian J Infect Dis* in press

8 **Beier FJ.** [Risk of endangering patients by hepatitis B infected surgeons: monitoring the health of medical personnel in hospitals must be evaluated]. *Gesundheitswesen* 2000; **62**: 64-70

9 **Luksamijarulkul P**, Maneesri P, Kittigul L. Hepatitis B seroprevalence and risk factors among school-age children in a low socioeconomic community, Bangkok. *Asia Pac J Public Health* 1995; **8**: 158-161

10 **Hann HW,** Jonsson Funk ML, Rosenberg DM, Davis R. Factors associated with response to lamivudine: Retrospective study in a tertiary care clinic serving patients with chronic hepatitis B. *J Gastroenterol Hepatol* 2005; **20**: 433-440

11 **Yao G,** Wang B, Cui Z, Yao J, Zeng M. A randomized double-blind placebo-controlled study of lamivudine in the treatment of patients with chronic hepatitis B virus infection. *Chin Med J (Engl)* 1999; **112**: 387-391

12 **Khokhar N,** Gill ML, Alam AY. Treatment of chronic hepatitis B with lamivudine. *J Coll Physicians Surg Pak* 2005; **15**: 78-80

13 **Ferenci P**. Treatment of chronic viral hepatitis. *Best Pract Res Clin Gastroenterol* 2004; **18** Suppl: 113-120

14 **Dienstag JL,** Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256-1263

15 **Lai CL,** Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61-68

16 **Schalm SW,** Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. *Gut* 2000; **46**: 562-568

17 **Perrillo RP,** Lai CL, Liaw YF, Dienstag JL, Schiff ER, Schalm SW, Heathcote EJ, Brown NA, Atkins M, Woessner M, Gardner SD. Predictors of HBeAg loss after lamivudine treatment for chronic hepatitis B. *Hepatology* 2002; **36**: 186-194

18 **Sokal E.** Lamivudine for the treatment of chronic hepatitis B. *Expert Opin Pharmacother* 2002; **3**: 329-339

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Comparison of invasive methods and two different stool antigen tests for diagnosis of *H pylori* infection in patients with gastric bleeding

Ebru Demiray, Özlem Yılmaz, Cihat Şarkış, Müjde Soytürk, İlkay Şimşek

Ebru Demiray, Özlem Yılmaz, Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Dokuz Eylül University, Inciraltı 35340 Izmir, Turkey
Cihat Şarkış, Müjde Soytürk, İlkay Şimşek, Department of Gastroenterology, Faculty of Medicine, Dokuz Eylül University, Inciraltı 35340 Izmir, Turkey
Correspondence to: Dr. Özlem Yilmaz, Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Dokuz Eylül University, Inciraltı 35340 Izmir, Turkey. ozlem.yilmaz@deu.edu.tr
Telephone: +90-232-4124506
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Abstract

AIM: To compare two different *H pylori* stool antigen tests as noninvasive diagnostic methods.

METHODS: The study population consisted of 22 upper gastrointestinal system bleeding patients. Urea breath test (UBT), rapid urease test (RUT) and histopathological examination were applied to all patients. Stool specimens from these patients were examined by rapid STRİP!HpSA and one step simple *H pylori* antigen cassette test for the detection of *H pylori* antigens.

RESULTS: For these 22 patients, 15 (68.2%) were diagnosed as positive and seven (31.8%) were diagnosed negative for *H pylori* infection by the gold standard methods. Whereas 10 (45.5%) were positive and 12 (54.5%) were diagnosed negative by the rapid STRİP!HpSA test. The sensitivity, specificity, positive and negative predictive values were 60%, 86%, 90% and 50%, respectively. When compared to the gold standard methods, these differences were not significant. However, six patients (27.3%) were positive, and 16 (72.7%) were negative by the simple *H pylori* stool antigen cassette test. The sensitivity, specificity, positive and negative predictive values were 33%, 86%, 83% and 38%, respectively. Compared to the gold standard methods, the simple *H pylori* stool antigen cassette test results were significantly different (P = 0.012).

CONCLUSION: Rapid STRİP!HpSA test could be used as a routine diagnostic tool in the microbiology laboratory for assessing clinical significance and eradication control of *H pylori* in upper gastrointestinal system bleeding patients.

INTRODUCTION

H pylori is a microaerophilic gram-negative spiral shaped bacterium[1-3]. It is recognized as the major cause of gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma, however little is known about its role in functional dyspepsia[4,5]. *H pylori* colonizes the gastric mucosa and attaches to the gastric epithelial cells[6]. The prevalence of *H pylori* infection is 70%-90% in developing countries and 25%-50% in developed countries[2,7]. Person-to-person spread is the most probable mode of transmission. Faecal-oral and oral-oral transmissions have been reported[8]. Chronic *H pylori* infections of the stomach are increasingly recognized as a major risk factor for the development of gastroduodenal disease. *H pylori* can be detected by noninvasive and invasive methods, the latter requiring endoscopy. Noninvasive testing for *H pylori* can be done by measuring exhaled ^{13}C urea breath test (UBT), by serology, by stool antigen tests, by a simple stool PCR and by analyzing body materials such as saliva and urine[2,9]. Although histopathology and culture of the organism, which are not easily and routinely performed, is considered the gold standard for the diagnosis of *H pylori* infection, we need rapid, accurate and reliable noninvasive methods[2].

In the case of upper gastrointestinal bleeding which is a major cause of morbidity, mortality and medical care costs, peptic ulcer is the most frequent source of bleeding in these patients. Treatment of *H pylori* infection is more effective than antisecretory non-eradicating therapy in preventing recurrent upper gastrointestinal bleeding from peptic ulcer. Consequently, all patients with peptic ulcer

bleeding should be tested for *H pylori*, and eradication therapy should be prescribed to infected patients[10].

In the presence of upper gastrointestinal bleeding, the diagnosis of *H pylori* infection may be compromised. The UBT is responsible for a high number of false negative results when it is used to diagnose *H pylori* in patients with upper gastrointestinal bleeding. Coagulation disorders or anticoagulation may prevent a biopsy being taken. The UBT may not be feasible in patients on artificial respiration, or in the presence of impaired consciousness or acute abdominal disease. Therefore, it is suggested that noninvasive methods, such as serology or UBT, be used to identify *H pylori* infection in these patients. In some cases, the indication for *H pylori* eradication therapy is based only on a serological test. Serology alone, however, is a rather inaccurate diagnosis method[10-12]. An ideal noninvasive test for *H pylori* infection should be safe and acceptable to patients, inexpensive and easy to perform, and with a high degree of sensitivity and specificity[13].

The diagnostic role of HpSA test and simple *H pylori* antigen cassette test in patients with upper gastrointestinal bleeding remains unclear. Only a few reports have discussed the results of the HpSA test and simple *H pylori* antigen cassette test in these patients[10-12,14]. The aim of this study was to evaluate diagnostic accuracy of stool antigen tests of rapid STRİP!HpSA and simple *H pylori* antigen cassette test other than HpSA in patients with upper gastrointestinal bleeding. We also compared two different *H pylori* stool antigen tests as noninvasive diagnostic methods with UBT, RUT and histopathology as gold standard methods.

MATERIALS AND METHODS

Patients

The study population consisted of 22 upper gastrointestinal system bleeding patients (13 males, 9 females; mean age, 58 ± 18 years; age range, 20 to 86 years) between September 2004 and January 2005. UBT (Infai, Germany), rapid urease test (RUT) and histopathological examination were applied to all patients.

The diagnosis of *H pylori* was defined as positive for UBT alone or for histopathology and (or) RUT results defined as gold standards. A patient was classified as *H pylori* negative when UBT and (or) histopathological examination and urease test were both negative.

Endoscopy and biopsy sampling

Two antrum and two corpus biopsy specimens were taken from each patient undergoing upper endoscopy from the same location in the stomach: one from the antrum and the corpus. One of these was used for the rapid urease test and the other two were immediately fixed and transported in 10% phosphate-buffered formalin solution for histopathologic examination.

Histopathologic examination of biopsy specimens

Paraffin-embedded gastric biopsy specimens were routinely processed. Haematoxylin-eosin, Alcian blue and Giemsa stains were used for morphologic examination of *Helicobacter*-like organisms (HLO) and updated Sydney system was used[15]. Histopathology was performed by a specialized pathologist.

Stool specimens

Stool specimens from these patients were collected and were kept at -20℃ until used. They were examined by rapid STRİP!HpSA (Meridian Bioscience Europe) and one step simple *H pylori* antigen cassette test (Linear Chemicals, S.L. Spain) for the detection of *H pylori* antigens. Both tests were performed in accordance with the manufacturer's specifications.

Stool antigen tests

Rapid STRİP!HpSA (Meridian Bioscience Europe) is a rapid immunoassay using a monoclonal anti-*H pylori* antibody on a strip for the detection of *H pylori* infections in stool specimens. The strip is introduced in a tube containing diluted patient samples and the appearance of a pink-red line in the reading area indicates a positive result after 5 min of incubation at room temperature. A positive test result is evaluated according to the blue band (control line), a distinguishable pink-red band (test line) also appears across the white central zone of the reaction strip. Any pink-red line, even very weak, must be considered as a positive result. By contrast, the sample is considered negative when only one blue coloured band (control line) appears across the white central area of the reaction strip.

Simple *H pylori* antigen cassette test (Linear Chemicals, S.L. Spain) is a rapid immunochromatographic test using a single monoclonal antibody. A diluted fecal sample is placed in an immunochromatographic support and the result is read after 5 min. A positive test will display a red line in the reading area next to a blue control line. Even a minimal trace of a red line was considered positive. By contrast, the sample is considered negative when only the control blue line develops. If no line appears in the reading area the test is considered null[14,16].

RESULTS

The diagnosis of *H pylori* was defined as positive for UBT alone or for histopathology and (or) RUT results defined by gold standard methods. A patient was classified as *H pylori* negative when histopathological examination and urease test were both negative.

For those 22 patients, 15 (68.2%) were diagnosed as positive and seven (31.8%) negative for *H pylori* infection by the gold standard methods. Meanwhile, 10 (45.5%) were positive and 12 (54.5%) were negative by the rapid STRİP!HpSA test. The sensitivity, specificity, positive and negative predictive values were 60%, 86%, 90% and 50%, respectively. When compared to the gold standard methods, these differences were not statistically significant. However, six patients (27.3%) were positive, and 16 (72.7%) were negative by the simple *H pylori* stool antigen cassette test. The sensitivity, specificity, positive and negative predictive values were 33%, 86%, 83% and 38%, respectively. Compared to the results by the gold standard methods, the simple *H pylori* stool antigen cassette test

Table 1 Results of invasive and noninvasive methods for diagnosis of *H pylori* infection in patients with gastric bleeding

	Positive *n* (%)	Negative *n* (%)	False positive *n* (%)	False negative *n* (%)
Urea breath test	12 (54.54)	10 (45.45)	2 (9.09)	2 (9.09)
Rapid urease test and/or histopathology	15 (68,18)	7 (31.82)	-	-
Rapid STRİP!HpSA stool antigen test	10 (45.45)	12 (54.54)	1 (4.54)	6 (27.27)
Simple *H pylori* stool antigen cassette test	6 (27.27)	16 (72.73)	1 (4.54)	10 (45.45)

results were statistically different ($P = 0.012$) (Table 1).

DISCUSSION

Many studies have described the use of ELISA-based HpSA stool antigen kits with either polyclonal or monoclonal antibodies, for diagnosis of *H pylori* infections. In this study we evaluated the diagnostic accuracy of two non-ELISA-based kits, rapid STRİP!HpSA and simple *H pylori* antigen cassette, in patients with upper gastrointestinal bleeding.

Meridian Bioscience, Inc. introduced the concept of detecting *H pylori* antigens in stool specimens, with a microtiter based immunoassay, in 1997. Primer Platinum HpSA, after extensive evaluation, was accepted as an accurate tool for non-invasive *H pylori* infection diagnosis[17-19]. Recent official European Guidelines recommend the use of either stool antigen or urea breath test for diagnosis and confirmation of eradication four weeks after the end of the treatment[20].

To our knowledge, three recent studies have evaluated the HpSA test in the presence of gastrointestinal bleeding: Gisbert *et al* ($n = 34$ hospitalized patients; sensitivity of polyclonal ELISA, monoclonal ELISA and monoclonal immunochromatographic test was 74%, 94%, 60%, respectively), Lin *et al* ($n = 93$ patients with bleeding peptic ulcers and 59 patients with nonbleeding peptic ulcers; sensitivity 82% and specificity 68%) and Peitz *et al* ($n = 114$ patients; the sensitivity 84% and specificity 90%)[10-12].

In another study, Erzin *et al* compared two different stool antigen tests for the primary diagnosis of *H pylori* infection in Turkish patients with dyspepsia. A total of 151 patients who were referred to the endoscopy unit were included. They used FemtoLab *H pylori* enzyme immunoassay and Premier Platinum. The sensitivity and specificity of the monoclonal FemtoLab *H pylori* were 93% and 90% respectively, and of the polyclonal Premier Platinum HpSA were 84% and 67%, respectively. They concluded that Femtolab *H pylori* was an excellent tool for primary diagnosis of *H pylori* in Turkish patients with dyspepsia[21].

Still in another study, Kato *et al* compared rapid lateral flow stool antigen immunoassay (LFI) and stool antigen enzyme immunoassay (EIA) for the diagnosis of *H pylori* infection in children. One hundred and eighty-two children and adolescents were studied. The sensitivity, specificity and accuracy of the LFI method were 90.6%, 95.8% and 94% respectively and for the EIA method, sensitivity, specificity and accuracy were 96.8%, 99.2% and 98.3%, respectively[22].

Islam *et al* assessed the performance of the HpSA for the diagnosis of *H pylori* infection and confirming post-therapy eradication compared to generally well accepted clinical reference standard. HpSA was used for the 127 patients. The sensitivity and specificity of HpSA were 67% and 100%, respectively. As a result, HpSA was found to be a reasonably useful diagnostic test for *H pylori*. The HpSA may prove to be useful for the primary care physicians as part of the test-and-treat strategy for dyspepsia, but this may need further study[23].

Ito *et al* investigated the clinical usefulness of HpSA test for the evaluation of the success of eradication therapy by comparing it with the UBT. A total of 105 patients with *H pylori* infection were enrolled in that study. The diagnostic accuracy of the UBT and the HpSA test was 94.3% and 97.1%, respectively. As a result, HpSA is a very useful and non-invasive diagnostic tool for the evaluation of eradication therapy of *H pylori*. A combination of the HpSA test and the UBT is very practical in the clinical evaluation of eradication therapy of *H pylori* [24].

Manes *et al* compared the accuracy of HpSA, FemtoLab and UBT in the assessment of eradication of *H pylori* infection 4-8 wk after the completion of antibiotic treatment. Three hundred and forty-six patients were studied. The sensitivity and specificity of HpSA were 73.4% and 97.8%, respectively. The sensitivity and specificity of FemtoLab were 88.3% and 97.8%, respectively. They concluded that both the new stool antigen tests, although less accurate, may represent valuable alternatives to UBT since they were cheap and easy to perform and did not need the use of expensive isotope ratio mass spectrometers. Thus, due to its high level of sensitivity, the new monoclonal stool test could be preferred for the post-eradication setting of *H pylori* infection[25].

Five recent studies done by Shaikh *et al* ($n = 86$ children; sensitivity 76% and specificity 61%), Raguza *et al* (133 children; sensitivity 94.6% and specificity 96.6%), de Carvalho Costa Cardinali *et al* (161 children; sensitivity 96.9% and specificity 100%), van Doorn *et al* (106 children; sensitivity 100% and specificity 92%) and Elitsur *et al* (121 children; sensitivity 67% and specificity 99%) evaluated the HpSA test in asymptomatic children with *H pylori* infection compared to other methods. It was demonstrated that the commercial polyclonal HpSA test can not replace histopathologic findings as the best standard for the diagnosis of *H pylori* infection in children[26-30].

Syam *et al* evaluated the HpSA for the detection of *H pylori* infection in 63 dyspeptic patients. The sensitivity and specificity of HpSA were 66.7% and 78.9%, respectively. They concluded that HpSA stool test may be useful for the primary diagnosis of *H pylori* infection in peptic ulcer[31].

Arıkan *et al* conducted a prospective study to examine the reliability of the HpSA test. The HpSA test had a 91% sensitivity and 83% specificity. HpSA test proved to be as reliable as pathological examination for confirming the existence of *H pylori* in humans. Thus, the HpSA test was a useful method for detecting *H pylori* in patients for whom

endoscopy was not indicated[32].

Lopez *et al* compared the efficacy of several non-invasive methods and assessed comparative reliability of the stool tests. Eighty-six patients were applied FemtoLab, HpSA and Simple *H pylori* tests. The sensitivity and specificity of HpSA were 58% and 96%, respectively and the sensitivity and specificity of simple *H pylori* were 61% and 78%, respectively. According to the results, they suggested that UBT was the most reliable diagnostic examination for determining *H pylori* status in patients with chronic renal failure on dialysis because stool tests showed heterogeneous results[14].

Inelmen *et al* evaluated the accuracy of HpSA in the diagnosis of *H pylori* infection in 85 elderly patients affected by medication. Among 56 patients who were not taking PPIs, the sensitivity and specificity of the HpSA test were 76% and 93%, respectively. Among 29 patients who had received pharmacological therapy with PPIs, the sensitivity and specificity of HpSA test were, respectively, 82% and 83%. They concluded that HpSA was a useful test in elderly people. The test was easy, simple to perform and non-invasive[13].

Chisholm *et al* conducted a comparative evaluation of the performances of Premier Platinum HpSA ELISA, Amplified IDEIA HpStAR ELISA and ImmunoCard STAT!HpSA kits. ImmunoCard STAT!HpSA was demonstrated easier to perform than ELISA and was more sensitive than the HpSA kit. Compared with the IDEIA HpStAR kit, the ImmunoCard test was less sensitive (87.8% versus 95.9%, respectively) and specific (89.4% versus 100.0%, respectively). The Amplified IDEIA HpStAR kit was the most sensitive and specific of the three tests and it was available for pre-treatment, noninvasive detection of *H pylori* in stool samples in an English adult dyspeptic population[33].

Previously we studied forty adult Turkish dyspeptic patients. Two antrum and corpus biopsies were taken from each patient and RUT and histopathology were applied to all patients as gold standard methods for the diagnosis of *H pylori* infection. Stool specimens were examined by polyclonal Premier Platinum HpSA and simple *H pylori* cassette test before and after the eradication therapy. When we compared with gold standard methods, the diagnostic accuracy of Premier Platinum HpSA and simple *H pylori* cassette tests were 75% and 87.5%, respectively.

In that study the HpSA test was polyclonal test but we wanted to compare it with the available monoclonal Simple *H pylori* cassette test[34]. The new Rapid STRİP!HpSA test was also used in this group of patients (unpublished data). As no results have been published to date on the rapid STRİP!HpSA test and the simple *H pylori* stool antigen cassette test, we undertook the present study. The simple *H pylori* stool antigen cassette test, although easy to use, gave a sensitivity that was too low (33%) for the reliable diagnosis of *H pylori* infection. However, the rapid STRİP!HpSA test, which was also convenient to use, was more sensitive (60%). We conclude that because it is a monoclonal test, rapid STRİP!HpSA, can be used as a routine diagnostic tool in the microbiology laboratory for assessing clinical significance and eradication control of *H pylori* in upper gastrointestinal bleeding patients.

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REFERENCES

1 **Vinette KM**, Gibney KM, Proujansky R, Fawcett PT. Comparison of PCR and clinical laboratory tests for diagnosing H. pylori infection in pediatric patients. *BMC Microbiol* 2004; **4**: 5
2 **Kabir S**. Detection of Helicobacter pylori in faeces by culture, PCR and enzyme immunoassay. *J Med Microbiol* 2001; **50**: 1021-1029
3 **Kabir S**. Review article: clinic-based testing for Helicobacter pylori infection by enzyme immunoassay of faeces, urine and saliva. *Aliment Pharmacol Ther* 2003; **17**: 1345-1354
4 **Makristathis A**, Barousch W, Pasching E, Binder C, Kuderna C, Apfalter P, Rotter ML, Hirschl AM. Two enzyme immunoassays and PCR for detection of Helicobacter pylori in stool specimens from pediatric patients before and after eradication therapy. *J Clin Microbiol* 2000; **38**: 3710-3714
5 **Braden B**, Caspary WF. Detection of Helicobacter pylori infection: when to perform which test? *Ann Med* 2001; **33**: 91-97
6 **Ruiz-Bustos E**, Ochoa JL, Wadström T, Ascencio F. Isolation and characterisation of putative adhesins from Helicobacter pylori with affinity for heparan sulphate proteoglycan. *J Med Microbiol* 2001; **50**: 215-222
7 **Dunn BE**, Cohen H, Blaser MJ. Helicobacter pylori. *Clin Microbiol Rev* 1997; **10**: 720-741
8 **Allaker RP**, Young KA, Hardie JM, Domizio P, Meadows NJ. Prevalence of helicobacter pylori at oral and gastrointestinal sites in children: evidence for possible oral-to-oral transmission. *J Med Microbiol* 2002; **51**: 312-317
9 **Sen N**, Yilmaz O, Simşek I, Küpelioğlu AA, Ellidokuz H. Detection of Helicobacter pylori DNA by a simple stool PCR method in adult dyspeptic patients. *Helicobacter* 2005; **10**: 353-359
10 **Gisbert JP**, Trapero M, Calvet X, Mendoza J, Quesada M, Güell M, Pajares JM. Evaluation of three different tests for the detection of stool antigens to diagnose Helicobacter pylori infection in patients with upper gastrointestinal bleeding. *Aliment Pharmacol Ther* 2004; **19**: 923-929
11 **Peitz U**, Leodolter A, Kahl S, Agha-Amiri K, Wex T, Wolle K, Günther T, Steinbrink B, Malfertheiner P. Antigen stool test for assessment of Helicobacter pylori infection in patients with upper gastrointestinal bleeding. *Aliment Pharmacol Ther* 2003; **17**: 1075-1084
12 **Lin HJ**, Lo WC, Perng CL, Li AF, Tseng GY, Sun IC, Ou YH. Helicobacter pylori stool antigen test in patients with bleeding peptic ulcers. *Helicobacter* 2004; **9**: 663-668
13 **Inelmen EM**, Gasparini G, Sergi G, Enzi G. Evaluation of Helicobacter pylori with a stool antigen assay in frail, elderly patients. *Scand J Gastroenterol* 2005; **40**: 794-799
14 **López T**, Quesada M, Almirall J, Sanfeliu I, Segura F, Calvet X. Usefulness of non-invasive tests for diagnosing Helicobacter pylori infection in patients undergoing dialysis for chronic renal failure. *Helicobacter* 2004; **9**: 674-680
15 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181
16 **Krogfelt KA**, Lehours P, Mégraud F. Diagnosis of Helicobacter pylori Infection. *Helicobacter* 2005; **10** Suppl 1: 5-13
17 **Vaira D**, Malfertheiner P, Mégraud F, Axon AT, Deltenre M, Hirschl AM, Gasbarrini G, O'Morain C, Garcia JM, Quina M, Tytgat GN. Diagnosis of Helicobacter pylori infection with a new non-invasive antigen-based assay. HpSA European study group. *Lancet* 1999; **354**: 30-33
18 **Vaira D**, Malfertheiner P, Mégraud F, Axon AT, Deltenre M, Gasbarrini G, O'Morain C, Pajares Garcia JM, Quina M,

Tytgat GN. Noninvasive antigen-based assay for assessing Helicobacter pylori eradication: a European multicenter study. The European Helicobacter pylori HpSA Study Group. *Am J Gastroenterol* 2000; **95**: 925-929

19 **Vaira D**, Vakil N. Blood, urine, stool, breath, money, and Helicobacter pylori. *Gut* 2001; **48**: 287-289

20 **Malfertheiner P**, Mégraud F, O'Morain C, Hungin AP, Jones R, Axon A, Graham DY, Tytgat G. Current concepts in the management of Helicobacter pylori infection--the Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* 2002; **16**: 167-180

21 **Erzin Y**, Altun S, Dobrucali A, Aslan M, Erdamar S, Dirican A, Kocazeybek B. Comparison of two different stool antigen tests for the primary diagnosis of Helicobacter pylori infection in turkish patients with dyspepsia. *Helicobacter* 2004; **9**: 657-662

22 **Kato S**, Ozawa K, Okuda M, Nakayama Y, Yoshimura N, Konno M, Minoura T, Iinuma K. Multicenter comparison of rapid lateral flow stool antigen immunoassay and stool antigen enzyme immunoassay for the diagnosis of Helicobacter pylori infection in children. *Helicobacter* 2004; **9**: 669-673

23 **Islam S**, Weilert F, Babington R, Dickson G, Smith AC. Stool antigen testing for the diagnosis and confirmation of eradication of Helicobacter pylori infection: a prospective blinded trial. *Intern Med J* 2005; **35**: 526-529

24 **Ito M**, Tanaka S, Kim S, Tahara K, Kawamura Y, Sumii M, Takehara Y, Hayashi K, Okamoto E, Kunihiro M, Kunita T, Imagawa S, Takata S, Ueda H, Egi Y, Hiyama T, Ueno Y, Kitadai Y, Yoshihara M, Chayama K. A combination of the Helicobacter pylori stool antigen test and urea breath test is useful for clinical evaluation of eradication therapy: a multicenter study. *J Gastroenterol Hepatol* 2005; **20**: 1241-1245

25 **Manes G**, Zanetti MV, Piccirillo MM, Lombardi G, Balzano A, Pieramico O. Accuracy of a new monoclonal stool antigen test in post-eradication assessment of Helicobacter pylori infection: comparison with the polyclonal stool antigen test and urea breath test. *Dig Liver Dis* 2005; **37**: 751-755

26 **Shaikh S**, Khaled MA, Islam A, Kurpad AV, Mahalanabis D. Evaluation of stool antigen test for Helicobacter pylori infection in asymptomatic children from a developing country using 13C-urea breath test as a standard. *J Pediatr Gastroenterol Nutr* 2005; **40**: 552-554

27 **Raguza D**, Granato CF, Kawakami E. Evaluation of the stool antigen test for Helicobacter pylori in children and adolescents. *Dig Dis Sci* 2005; **50**: 453-457

28 **de Carvalho Costa Cardinali L**, Rocha GA, Rocha AM, de Moura SB, de Figueiredo Soares T, Esteves AM, Nogueira AM, Cabral MM, de Carvalho AS, Bitencourt P, Ferreira A, Queiroz DM. Evaluation of [13C]urea breath test and Helicobacter pylori stool antigen test for diagnosis of *H. pylori* infection in children from a developing country. *J Clin Microbiol* 2003; **41**: 3334-3335

29 **van Doorn OJ**, Bosman DK, van't Hoff BW, Taminiau JA, ten Kate FJ, van der Ende A. Helicobacter pylori Stool Antigen test: a reliable non-invasive test for the diagnosis of Helicobacter pylori infection in children. *Eur J Gastroenterol Hepatol* 2001; **13**: 1061-1065

30 **Elitsur Y**, Lawrence Z, Hill I. Stool antigen test for diagnosis of Helicobacter pylori infection in children with symptomatic disease: a prospective study. *J Pediatr Gastroenterol Nutr* 2004; **39**: 64-67

31 **Syam AF**, Rani AA, Abdullah M, Manan C, Makmun D, Simadibrata M, Djojoningrat D, Sato T. Accuracy of Helicobacter pylori stool antigen for the detection of Helicobacter pylori infection in dyspeptic patients. *World J Gastroenterol* 2005; **11**: 386-388

32 **Arikan S**, Kocakusak A, Barut G, Sengoz G, Yucel AF, Gokturk K. Helicobacter pylori stool antigen test: results of a prospective study. *Surg Today* 2004; **34**: 318-322

33 **Chisholm SA**, Watson CL, Teare EL, Saverymuttu S, Owen RJ. Non-invasive diagnosis of Helicobacter pylori infection in adult dyspeptic patients by stool antigen detection: does the rapid immunochromatography test provide a reliable alternative to conventional ELISA kits? *J Med Microbiol* 2004; **53**: 623-627

34 **Yılmaz Ö**, Şen N, Soytürk M, Tankurt İE. Helicobacter pylori tanısında iki farklı dışkı antijen testinin karşılaştırılması. İstanbul: KLİMİK 2003 XI. Türk Klinik Mikrobiyoloji ve İnfeksiyon Hastalıkları Kongresi 2003; **292**: 03/03

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PO Box 2345, Beijing 100023, China
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wjg@wjgnet.com

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RAPID COMMUNICATION

Incidence and management of colonoscopic perforations: 8 years' experience

Hagit Tulchinsky, Osnat Madhala-Givon, Nir Wasserberg, Shlomo Lelcuk, Yaron Niv

Hagit Tulchinsky, Osnat Madhala-Givon, Nir Wasserberg, Shlomo Lelcuk, Department of Surgery B, Rabin Medical Center, Beilinson Campus, Petah Tiqva, and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
Yaron Niv, Department of Gastroenterology, Rabin Medical Center, Beilinson Campus, Petah Tiqva, and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
Correspondence to: Hagit Tulchinsky, MD, Department of Surgery B, Sourasky Medical Center, 6 Veizman St., Tel Aviv 64239, Israel. hagitt@tasmc.health.gov.il
Telephone: +972-3-6973209 Fax: +972-3-6974635
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Abstract

AIM: To review the experience of a major medical teaching center with diagnostic and therapeutic colonoscopies and to assess the incidence and management of related colonic perforations.

METHODS: All colonoscopies performed between January 1994 and December 2001 were studied. Data on patients, colonoscopic reports and procedure-related complications were collected from the departmental computerized database. The medical records of the patients with post procedural colonic perforation were reviewed.

RESULTS: A total of 12 067 colonoscopies were performed during the 8 years of the study. Seven colonoscopic perforations (4 females, 3 males) were diagnosed (0.058%). Five occurred during diagnostic and two during therapeutic colonoscopy. Six were suspected during or immediately after colonoscopy. All except one had signs of diffuse tenderness and underwent immediate operation with primary repair done in 4 patients. No deaths were reported.

CONCLUSION: Perforation rate during colonoscopy is low. Nevertheless, it is a serious complication and its early recognition and treatment are essential to optimize outcome. In patients with diffuse peritonitis early operative intervention makes primary repair a safe option.

Key words: Colonoscopy; Complications; Perforation; Polypectomy; Management

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INTRODUCTION

Since first introduction in 1969[1] flexible colonoscopy has been accepted as the best method for the diagnosis, treatment and follow-up of colorectal pathologies. Nevertheless, being an invasive procedure it harbors major risks of bleeding, perforation and even death[2-4]. The incidence of perforation is 0.2% to 0.4% for diagnostic colonoscopy and 0.3% to 1.0% with polypectomy[2,5,6]. Recent large series have reported lower perforation rates of 0.002% to 0.19%[7-10]. The aim of the present study was to review the experience of a major university affiliated medical center with colonoscopy and to assess the incidence of perforations and their management.

MATERIALS AND METHODS

A total of 12067 colonoscopies were performed between January 1994 and December 2001. Data on patients undergoing colonoscopy was entered into a computerized database and included demographic patient information and detailed colonoscopic reports. A retrospective review of the medical records of all patients diagnosed with colonic perforation after colonoscopy was performed. The following parameters were analyzed: patient age and sex, background disease, laboratory work-up, indication to endoscopy, interval from the procedure to the diagnosis of perforation, clinical presentation, location, management, and outcome of the perforation.

RESULTS

Of 12 067 colonoscopies performed, seven were associated with colonic perforation. There were 4 women and 3 men with a mean age of 70 years (range 31-80) (Table 1). The procedure was done on an outpatient basis in all cases. Five perforations occurred during diagnostic colonoscopy, one during polypectomy and one during electrocoagulation of an arteriovenous malformation. Six perforations were

identified during the procedure or immediately thereafter, and one patient who had a cecal polyp coagulated with hot biopsy forceps, was diagnosed about 24 h after the procedure. In 3 patients perforation was suspected when a hole in the intestinal wall was noted. All the patients had severe abdominal pain and distention. Plain abdominal roentgenograms performed in all 7 patients showed free intraperitoneal air in 4, retropneumoperitoneum in 2 and no abnormalities in one patient.

Six patients had, on examination, diffuse abdominal tenderness and underwent immediate operation. All five perforations that occurred during diagnostic colonoscopy were found to be in the sigmoid colon, and repair was achieved mostly by debridment and primary suture of the perforated site. The postoperative period was uneventful. One patient was treated nonoperatively with intravenous fluids, antibiotics and intestinal rest and was placed under close clinical observation. He was afebrile and had localized abdominal tenderness with no peritoneal signs or leukocytosis. The patient who was diagnosed and operated on about 24 h post colonoscopy had a wound infection. Median hospital stay of the operated group was 8 d (range 4-15). The patient who was treated nonoperatively was hospitalized for 13 d. There were no deaths.

DISCUSSION

Colonic perforation occurs rarely during colonoscopy but it is still considered a major complication[2-4]. During the 8-year period reviewed, there was a combined diagnostic and therapeutic colonoscopic perforation rate of 0.058%. This rate is lower than that in most of the published series[7-10]. The management of colonoscopic perforations may be conservative or surgical, and should be selective. The choice of treatment depends on the mechanism and size of the perforation, adequacy of bowel preparation, timing of diagnosis, the patient's clinical condition and the primary colonic pathology[9-16]. Table 1 summarizes some reports evaluating the incidence and management of colonoscopic perforations. The low morbidity rate in our series is probably attributed to the combination of supportive treatment and early surgical intervention, which resulted in no intraperitoneal contamination in 4 out of 5 patients, and therefore primary repair could be completed safely. Early surgical exploration in all patients with peritoneal irritation or free air on abdominal X-ray is recommended by other authors as well[10,17,18]. Farley *et al*[10] reported on 43 perforations among 57 028 colonoscopies (0.075%). Forty-two were treated by emergency laparotomy. Most patients underwent primary repair or limited resection with anastomosis. The authors concluded that in order to minimize morbidity and mortality prompt operative intervention is the best strategy in most patients. Dafnis *et al*[18] reported on 8 perforations in 6066 colonoscopies (0.13%). All patients underwent surgery. Most perforations were repaired by primary closure, and the postoperative course was uneventful in all patients.

Perforations occurring during diagnostic colonoscopy are due to direct mechanical penetration with the instrument tip, sharp flexion of the colonoscope, high pressure applied when a loop is formed or barotrauma as a result of aggressive gas insufflation[19,20]. The most common underlying cause in the present study was direct mechanical injury of the colonic wall by the colonoscope. It occurred in patients with diverticular disease or a strictured, severely diseased colonic segment. These risk factors are in accordance with those noted in the literature[21,22]. Perforations during therapeutic colonoscopy occur as a result of similar mechanisms, as well as from thermal or electrical injury, as in two cases in the present study[11,12]. The most frequent site of perforation was the sigmoid colon, as in other studies[10,13,18,23,24]. This may be explained by its anatomical characteristics of frequent redundancy or narrowing from diverticular disease or adhesions after previous pelvic operations[25].

In conclusion, although the rate of perforation during colonoscopy is low, it is a serious complication and its early recognition and treatment are essential to optimize outcome. Surgery is mandatory in all patients with generalized peritoneal irritation. Early operative intervention makes primary repair a good and safe option, with low morbidity and mortality, unless there is a colonic pathology that necessitates resection. Selected patients with localized peritoneal irritation can be managed nonoperatively.

Table 1 Reported colonoscopic perforation rates and management

Author	Time interval (yr)	No. of colono-scopies	Perforation	Mortality *n* (%)	
				Operative	Non op.
Anderson *et al*[7]	10	10 486	20 (0.19)	19 (1)	1 (1)
Araghizadeh *et al*[9]	30	34 620	31 (0.09)	20 (0)	11 (1)[1]
Farley *et al*[10]	16	57 028	43 + 2[2] (0.075)	42 (0)	3 (0)
Christie *et al*[12]	10	4784	7 (0.15)	2 (0)	5 (0)
Hall *et al*[14]	6 (4-15)	17 500	15 (0.09)	14 (0)	1 (0)
Jentschura *et al*[16]	9	29 695	31 (0.1)	24 (2)	7 (0)
Lo *et al*[17]	6	26 708	12 (0.04)	6 (1)	6 (0)
Dafnis *et al*[19]	17	6066	8 (0.1)	8 (0)	0
Carpio *et al*[25]	10	5424	14 (0.26)	8 (2)	6 (1)
Present study	8	12 067	7 (0.058)	6 (0)	1 (0)

[1]Three of 11 patients failed medical treatment and required surgery; [2]Two patients were treated after colonoscopy performed elsewhere; [3]Includes only colonoscopic polypectomies.

REFERENCES

1 **Wolff WI**, Shinya H. Colonofiberoscopy. *JAMA* 1971; **217**: 1509-1512

2 **Rogers BH**, Silvis SE, Nebel OT, Sugawa C, Mandelstam P. Complications of flexible fiberoptic colonoscopy and polypectomy. *Gastrointest Endosc* 1975; **22**: 73-77

3 **Smith LE**. Fiberoptic colonoscopy: complications of colonoscopy and polypectomy. *Dis Colon Rectum* 1976; **19**: 407-412

4 **Kavic SM**, Basson MD. Complications of endoscopy. *Am J Surg* 2001; **181**: 319-332

5 **Shahmir M**, Schuman BM. Complications of fiberoptic endoscopy. *Gastrointest Endosc* 1980; **26**: 86-91

6 **Mühldorfer SM**, Kekos G, Hahn EG, Ell C. Complications of therapeutic gastrointestinal endoscopy. *Endoscopy* 1992; **24**: 276-283

7 **Anderson ML**, Pasha TM, Leighton JA. Endoscopic perforation of the colon: lessons from a 10-year study. *Am J Gastroenterol* 2000; **95**: 3418-3422
8 **Gedebou TM**, Wong RA, Rappaport WD, Jaffe P, Kahsai D, Hunter GC. Clinical presentation and management of iatrogenic colon perforations. *Am J Surg* 1996; **172**: 454-457; discussion 457-458
9 **Araghizadeh FY**, Timmcke AE, Opelka FG, Hicks TC, Beck DE. Colonoscopic perforations. *Dis Colon Rectum* 2001; **44**: 713-716
10 **Farley DR**, Bannon MP, Zietlow SP, Pemberton JH, Ilstrup DM, Larson DR. Management of colonoscopic perforations. *Mayo Clin Proc* 1997; **72**: 729-733
11 **Christie JP**, Marrazzo J 3rd. "Mini-perforation" of the colon--not all postpolypectomy perforations require laparotomy. *Dis Colon Rectum* 1991; **34**: 132-135
12 **Wadas DD**, Sanowski RA. Complications of the hot biopsy forceps technique. *Gastrointest Endosc* 1988; **34**: 32-37
13 **Hall C**, Dorricott NJ, Donovan IA, Neoptolemos JP. Colon perforation during colonoscopy: surgical versus conservative management. *Br J Surg* 1991; **78**: 542-544
14 **Nelson RL**, Abcarian H, Prasad ML. Iatrogenic perforation of the colon and rectum. *Dis Colon Rectum* 1982; **25**: 305-308
15 **Jentschura D**, Raute M, Winter J, Henkel T, Kraus M, Manegold BC. Complications in endoscopy of the lower gastrointestinal tract. Therapy and prognosis. *Surg Endosc* 1994; **8**: 672-676
16 **Lo AY**, Beaton HL. Selective management of colonoscopic perforations. *J Am Coll Surg* 1994; **179**: 333-337
17 **Clements RH**, Jordan LM, Webb WA. Critical decisions in the management of endoscopic perforations of the colon. *Am Surg* 2000; **66**: 91-93
18 **Dafnis G**, Ekbom A, Pahlman L, Blomqvist P. Complications of diagnostic and therapeutic colonoscopy within a defined population in Sweden. *Gastrointest Endosc* 2001; **54**: 302-309
19 **Young HS**, Keeffe EB. Complications of gastrointestinal endoscopy. In: Sleisenger & Fordtran's gastrointestinal and liver diseases. 6th ed. Philadelphia: WB Saunders, 1998: 301-309
20 **Orsoni P**, Berdah S, Verrier C, Caamano A, Sastre B, Boutboul R, Grimaud JC, Picaud R. Colonic perforation due to colonoscopy: a retrospective study of 48 cases. *Endoscopy* 1997; **29**: 160-164
21 **Hunt RH**. Towards safer colonoscopy. *Gut* 1983; **24:** 371-375
22 **Macrae FA**, Tan KG, Williams CB. Towards safer colonoscopy: a report on the complications of 5000 diagnostic or therapeutic colonoscopies. *Gut* 1983; **24**: 376-383
23 **Carpio G**, Albu E, Gumbs MA, Gerst PH. Management of colonic perforation after colonoscopy. Report of three cases. *Dis Colon Rectum* 1989; **32**: 624-626
24 **Garbay JR**, Suc B, Rotman N, Fourtanier G, Escat J. Multicentre study of surgical complications of colonoscopy. *Br J Surg* 1996; **83**: 42-44
25 **Damore LJ 2nd**, Rantis PC, Vernava AM 3rd, Longo WE. Colonoscopic perforations. Etiology, diagnosis, and management. *Dis Colon Rectum* 1996; **39**: 1308-1314

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wjg@wjgnet.com

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RAPID COMMUNICATION

Liver microcirculation after hepatic artery embolization with degradable starch microspheres *in vivo*

Jian Wang, Satoru Murata, Tatsuo Kumazaki

Jian Wang, Department of Interventional Radiology and Vascular Surgery, The First Hospital of Peking University, 100034, Beijing, China
Satoru Murata, Tatsuo Kumazaki, Department of Radiology, Nippon Medical School, Tokyo, Japan
Correspondence to: Jian Wang, Department of Interventional Radiology and Vascular Surgery, the First Hospital of Peking University, 100034, Beijing, China. vanjian@sohu.com
Telephone: +86-10-66518436 Fax: +86-10-66518436
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Abstract

AIM: To observe the dynamic changes of liver microcirculation *in vivo* after arterial embolization with degradable starch microspheres (DSM).

METHODS: DSM were injected into the proper hepatic artery through a silastic tube inserted retrogradely in gastroduodenal artery (GDA) of SD rats. Fluorescent microscopy was used to evaluate the dynamic changes of blood flow through the terminal portal venules (TPVs), sinusoids and terminal hepatic venules (THVs). The movements of DSM debris were also recorded. Six hours after injection of DSM, percentages of THVs with completely stagnant blood flow were recorded.

RESULTS: Two phases of blood flow change were recorded. In phase one: after intra-arterial injection of DSM, slow or stagnant blood flow was immediately recorded in TPVs, sinusoids and THVs. This change was reversible, and blood flow resumed completely. In phase two: after phase one, blood flow in TPVs changed again and three patterns of blood flow were recorded. Six hours after DSM injection, 36.9% ± 9.2% of THVs were found with completely stagnant blood flow.

CONCLUSION: DSM can stop the microcirculatory blood flow in some areas of liver parenchyma. Liver parenchyma supplied by arteries with larger A-P shunt is considered at a higher risk of total microcirculatory blood stagnation after injection of DSM through hepatic artery.

Key words: Degradable starch microsphere; Hepatic microcirculation; Hepatic arteries; Fluorescence; Transarterial chemoembolization

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INTRODUCTION

It has been accepted that transarterial chemoembolization (TACE) is an effective method to treat the unresectable hepatocellular carcinoma (HCC). With the characteristics of long retention time in the tumor tissue, iodinated poppyseed oil (Lipiodol) has been frequently used as the embolization material in clinical practice[1-4]. Animal experiments demonstrated that after injection of iodized oil into the hepatic artery, small Lipiodol drops could be found in the terminal portal venules (TPVs), which was assumed passing through the pathway of arterial-portal anastomosis such as the peribiliary pluxes[5-7]. When Lipiodol drops flow into the sinusoids, they can severely occlude the blood flow, cause the stagnation of local microcirculation, and further lead to ischemic liver parenchyma injury[8,9]. Super-selective technique with microcatheter and guidewires has been considered as a safe and effective way to treat HCC. Under some conditions in which liver function is severely damaged or blood supply of HCC is so complex that it is impossible to super-select the tumor feeding arteries, TACE is developed. Degradable starch microspheres (DSM), a temporary artery embolizer has been increasingly used as an alternative to Lipiodol in some particular situations[10-12]. It has also been suggested that, when the tumor feeding artery cannot be super-selected by microcatheter and guidewire, one-shot injection of DSM before TACE can be regarded as a practical method to protect the tumor free liver tissue from the injury caused by Lipiodol inflow following TAE. To fully understand its effects on liver microcirculation, we injected DSM through proper hepatic artery of rats, and the dynamic changes of liver microcirculation were evaluated by *in vivo* fluorescent microscopic observations.

MATERIALS AND METHODS

Animal model

Ten Sprague-Dawley rats weighing 300 to 450 g were used in compliance with the regulations and the Guide for the Care and Use of Laboratory Animals. The animals were

Table 1 Blood flow in phase one in rats (min)

No.	Start of blood flow stagnation	Time of complete recovery of blood flow
1	1.1	10.9
2	1.4	13.1
3	2.6	15
4	1	14.9
5	1.6	9.82
6	2.3	11.5
7	2.8	13.2
8	3	14.5
9	2.8	14.3
10	1.6	9.47
mean ± SD	2.0 ± 0.8	12.7 ± 2.1

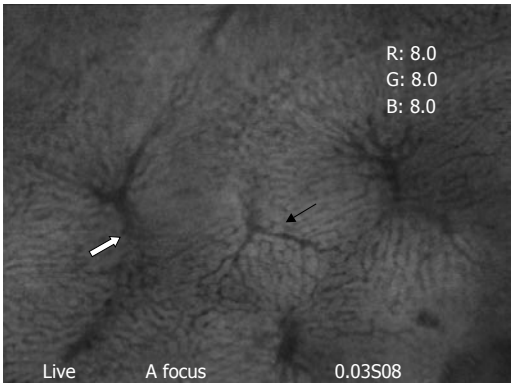

Figure 1 Normal microcirculation of the rat liver. Arrow: TPV, Opened arrow: THV.

fed with standard food pellets and tap water *ad libitum*. They were deprived of food but obtained free access to water for 12 h before the experiments. Anesthesia was performed by intra-peritoneal injection of 50 mg/kg sodium pentobarbital. The left femoral vein was cannulated with a 1F silastic tube (Natsume Corp. Tokyo, Japan) for additional anesthesia and liquid transfusion during the procedure. After a midline abdominal incision, the liver was carefully retracted to expose the gastroduodenal artery (GDA), which was catheterized by another 1F silastic tube (Natsume Corp., Tokyo, Japan) with its tip placed before the bifurcation that leads to the proper hepatic artery. The left lobe of liver was gently exteriorized and positioned over the window of the microscope stage. The liver parenchyma was covered with a small piece of plastic wrap; its surface was constantly irrigated with Ringer's solution at the body temperature.

Fluorescence microscopy

The exteriorized left liver lobe was transilluminated with monochromatic light generated by a prism monochromator equipped with a xenon lamp. Microscopic images of the microvasculatures were obtained with objective lenses (magnification, × 10, × 20) and an ocular lens (magnification, × 10). DSM (Yakult Honsha Co., Ltd., Tokyo, Japan) 12 mg in 0.2 mL was prepared in a 1 mL syringe and the solution was made uniform before injection. After infusion of 1 g/L fluorescent sodium 0.1 mL into the cannulated GDA, DSM was injected gently in one minute. The *in vivo* microscopic images of the following procedure were recorded on videotapes.

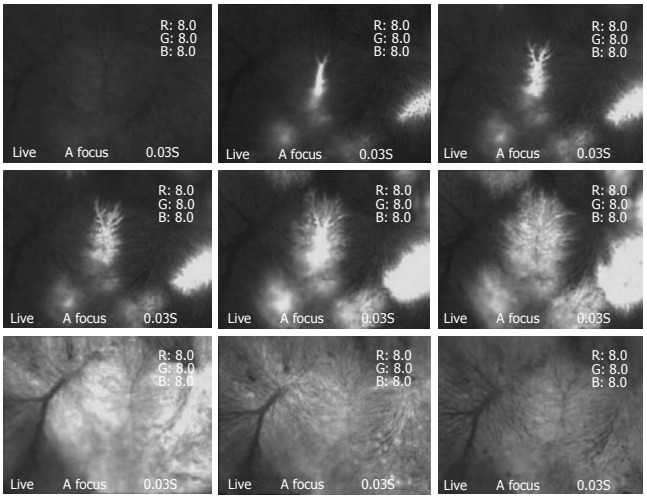

Figure 2 After infusion of fluorescent sodium from the GDA to proper hepatic artery, blood flow from TPVs to THVs through sinusoids can be clearly visualized.

In vivo evaluation

For each rat, areas with best visualization were selected for evaluation. Six hours later, 1 g/L fluorescent sodium 0.1 mL was infused through the cannulated left femoral vein to check the whole surface of liver lobe for a complete confirmation of the liver microcirculation. One hundred THVs were randomly selected during the horizontal and vertical movements of the microscope. THVs with completely stopped blood flow were statistically counted.

Statistical analysis

Data analysis was performed employing the Statistical Package for the Social Sciences Version 12.0 for Windows (SPSS© Inc., Chicago IL, USA). Results of the descriptive statistical analysis were presented as mean ± SD.

RESULTS

Clear images of the liver microcirculation (TPVs, sinusoids, and THVs) could be seen under *in vivo* fluorescent microscope (Figure 1). Blood flow from one TPV was drained through the sinusoids into several THVs; similarly, one particular THV provided venular drainage for several TPVs. Hepatic arterioles, the other afferent vessels in the liver, usually could not be visualized (Figure 2).

Blood flow in TPVs after DSM injection

Blood flow through TPVs demonstrated an immediate response after DSM injection. The speed of blood flow dropped dramatically at once. In 2.0 ± 0.8 min (Max 3 min), the blood flow in the observed area completely stopped. After that, blood flow through TPVs resumed gradually; 12.7 ± 2.1 min (Max 15 min) after injection of the DSM, blood flow through TPVs completely recovered (Table 1). No evidence of DSM or its disaggregated debris could be recorded within this time interval. For convenient explanation, we named this period as "phase one", and the

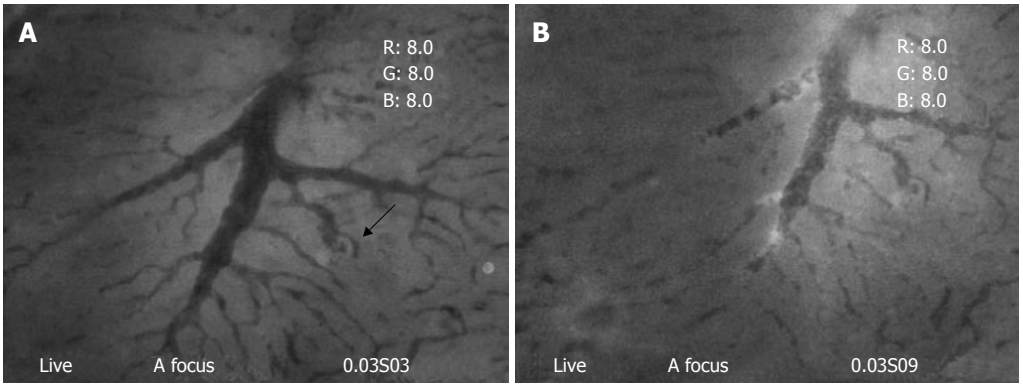

Figure 3 **A**: Small debris can enter sinusoids through TPV (arrow); **B**: The same TPV as image 3, debris of DSM continuously enter sinusoids through TPV, blood flow in TPV is intermittent but not stagnant.

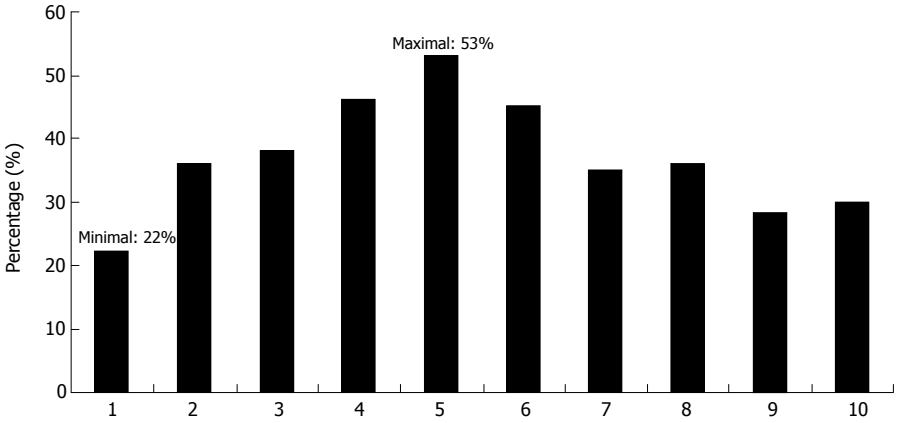

Figure 4 Percentage of the completely stagnant THVs 6 h after DSM injection in 10 rats. The minimal percentage is 22%, and the maximal is 53%.

blood flow changes afterward as "phase two". In phase two, three different types of blood flow changes in TPVs could be recorded.

Type one: The speed of blood flow slowed down again, and then completely stopped. This phenomenon could be found as early as 25 min after DSM injection and last for the whole procedure. The TPVs could never resume their blood flow during the observational period. DSM or its debris could be hardly found in TPVs in this type.

Type two: The speed of blood flow in TPVs decreased at different level, either slightly striking or some times intermittent, however the stagnation was not recorded during the whole observation time. Numerous small pieces of debris with irregular shapes could be found to flow through and drain into the distal sinusoids (Figure 3A and B).

Type three: In particular areas, TPVs kept a constant flow after phase one. The blood flow did not change during the whole procedure. DSM or its debris could not be recorded in these TPVs.

Blood flow in sinusoids and THVs

The blood flow through sinusoids and THVs followed the changes of TPVs in phase one. They also demonstrated a dramatic decrease of blood flow speed after DSM injection. Some even completely stopped. Nevertheless, it could be fully recovered.

During the following period, three kinds of blood flow, similar to that of the TPVs could also be recorded in sinusoids and THVs, ie, completely stagnant blood flow, intermittent and slow flow, normal flow.

DSM debris in the hepatic microvasculature

Twenty minutes (the earliest time) after injection of DSM, small pieces of debris could be found in some sinusoids. Some of the debris, with a relatively small size, could directly pass through the sinusoids and flow into THVs. Some debris, with larger size, could occlude the corresponding sinusoids. This occlusion was temporary; recanalization could be achieved by opening of collaterals or further distal movement of the disaggregated debris. The number of DSM debris reached a peak value 1 h after DSM injection. No DSM particles with the original size and shape could be found in sinusoids and THVs. Numerous disaggregated debris with small diameter entered the THVs.

Hepatic microcirculation after DSM injection

Six hours after DSM injection, the brightness of the liver surface was not uniform after infusion of fluorescent sodium through the femoral vein, suggesting the heterogeneous nature of the liver blood flow. Areas with completely stagnant blood flow in TPVs, THVs and sinusoids could be found sparsely distributed among the areas with normal or sluggish blood flow. Approximately 36.9% ± 9.2% of randomly selected THVs were found with completely stagnant blood flow (Figure 4).

DISCUSSION

DSM is a kind of embolization material that can temporarily occlude the vessels. The degradation of

DSM is considered to be caused by a combination of the chemical effects of amylase and the striking force of the vortexial arterial flow. Hepatic arterial perfusion is essential for an optimal sinusoidal function because it maintains transinusoidal pressure[13]. After intra-artery injection of DSM, immediately slow down or even stop of the blood flow in TPVs, sinusoids, and THVs could be found. It is considered that a sudden reduction of arterial blood flow is caused by numerous DSM casts embolization. The blocked blood flow could soon be resumed due to a compensatory increase of portal blood flow as a buffer response. This can explain the phenomenon of phase one which happened after the DSM injection. After a complete recovery of blood flow in phase one, presinusoidal A-P shunt[14-16] should be the reason for the appearance of three types of blood flow in phase two. We have found in our previous study that after intra-arterial injection of DSM, various sizes of DSM casts are formed inside the arterioles which can block the arterial blood flow. The proximal end of DSM casts will disaggregate under the pumping force of vortexial arterial blood flow. Debris with different sizes will be discharged at the proximal end and further occlude the branch of the original artery. We presume that A-P shunts have various size, some larger debris of the disaggregated DSM can pass through larger A-P shunts and reach the portal side that is proximal to TPVs. Debris accumulated at the portal side form a number of emboli. These emboli, if big enough, will completely shut the portal blood flow to distal TPVs and be harder to disaggregate because the pumping force of portal blood stream is much weaker than that of the arterial one. This means, at certain areas of liver parenchyma, both arterial and portal blood flows are stopped by DSM casts and the debris emboli. Few debris could be found in the distal TPVs because the proximal portion of TPVs was completely occluded by the debris emboli. This explains the type one phenomenon. For some small A-P shunt, only small-sized debris can pass through; and these smaller debris could partially occlude portal branch and was easily to be pushed distally. Some could reach TPVs and flow through sinusoids to THVs. This caused type two phenomenon. As for the type three phenomenon, it is considered that no debris of DSM entered portal site through A-P shunt.

After entering sinusoids, debris could occlude sinusoids**.** For a single sinusoid, the blood flow can be resumed either by further disaggregation of the debris or by opening of the small collaterals. Small DSM debris, when passing through sinusoid, will flow freely into THVs. Six hours after injection of DSM, we found 22%-53% (mean: 36.9% ± 9.2%) of THVs with totally stagnant blood flow. That means all sinusoids draining blood to these THVs had been stagnant in blood flow. The corresponding liver parenchyma received no fresh blood supply during this time. The cause of stagnant blood flow in sinusoids surrounding THVs is presumed at the presinusoidal level. We assume that the occlusion site was the presinusoidal portal vein with relatively larger diameter. After arterial embolization by DSM casts, the more bigger debris of DSM entered this portion through larger A-P shunt and accumulation of these debris formed intravascular emboli. Weak pumping force of portal blood flow could not disaggregate these emboli, and the amylase could take effects more slowly because little fresh blood flow could reach those emboli. With all those factors, the emboli could maintain stable during a fairly long period of time. Thus, a simultaneous blockade of the arterial and portal blood flow could lead to a completely stagnant blood flow in distal sinusoids. Because the amylase in blood flow will chemically disaggregate the DSM and its debris, whether the TPVs, sinusoids and THVs can resume their blood flow later needs to be further studied.

It is preliminarily confirmed in this study that DSM, with its degradation products, can enter portal vein through hepatic arterial injection. It can completely stop the microcirculatory blood flow in some areas of liver parenchyma. A-P shunt is considered to be a determining factor during the procedure. Liver parenchyma supplied by arteries with larger A-P shunt is presumed to have higher risk of total microcirculatory blood stagnation after injection of DSM through hepatic artery. Whether the use of DSM can provide protective effects during TACE awaits further evaluation.

REFERENCES

1 **Kim P**, Prapong W, Sze DY, So SK, Razavi MK. Treatment of hepatocellular carcinoma with sub-selective transcatheter arterial oily chemoinfusion. *Tech Vasc Interv Radiol* 2002; **5**: 127-131

2 **Lo CM**, Ngan H, Tso WK, Liu CL, Lam CM, Poon RT, Fan ST, Wong J. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology* 2002; **35**: 1164-1171

3 **Chen MS**, Li JQ, Zhang YQ, Lu LX, Zhang WZ, Yuan YF, Guo YP, Lin XJ, Li GH. High-dose iodized oil transcatheter arterial chemoembolization for patients with large hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 74-78

4 **Bhattacharya S**, Dusheiko GM. Treatment of unresectable hepatocellular carcinoma: targeted therapies using iodized oil. *Princess Takamatsu Symp* 1995; **25**: 253-264

5 **Kan Z**, Sato M, Ivancev K, Uchida B, Hedgpeth P, Lunderquist A, Rosch J, Yamada R. Distribution and effect of iodized poppyseed oil in the liver after hepatic artery embolization: experimental study in several animal species. *Radiology* 1993; **186**: 861-866

6 **Kan Z**, Wallace S. Sinusoidal embolization: impact of iodized oil on hepatic microcirculation. *J Vasc Interv Radiol* 1994; **5**: 881-886

7 **Kan Z**, Ivancev K, Hägerstrand I, Chuang VP, Lunderquist A. In vivo microscopy of the liver after injection of Lipiodol into the hepatic artery and portal vein in the rat. *Acta Radiol* 1989; **30**: 419-425

8 **Akashi Y**, Koreeda C, Mizuno T, Inoue K, Kawa SK, Tanaka Y. Hepatic parenchymal changes after the intraarterial injection of lipiodol in tumor-bearing rabbits. *Invest Radiol* 1993; **28**: 128-132

9 **Sato M**, Yamada R, Uchida B, Hedgepeth P, Rosch J. Effects of hepatic artery embolization with Lipiodol and gelatin sponge particles on normal swine liver. *Cardiovasc Intervent Radiol* 1993; **16**: 348-354

10 **Furuse J**, Ishii H, Satake M, Onaya H, Nose H, Mikami S, Sakai H, Mera K, Maru Y, Yoshino M. Pilot study of transcatheter arterial chemoembolization with degradable starch microspheres in patients with hepatocellular carcinoma. *Am J Clin Oncol* 2003; **26**: 159-164

11 **Kigami Y**, Kobayashi H, Umeoka S, Emoto T, Akuta K. Early effect of intra-arterial chemotherapy combined with degradable starch microspheres for malignant hepatic tumors.

Gan To Kagaku Ryoho 2003; **30**: 81-87

12 **Katsumata K**, Tomioka H, Sumi T, Yamasaki T, Takagi M, Kato F, Suzuki Y, Aoki T, Koyanagi Y. Liver metastasis of pancreatic cancer managed by intra-arterial infusion chemotherapy combined with degradable starch microspheres. *Int J Clin Oncol* 2003; **8**: 110-112

13 **Alexander B**, Cottam H, Naftalin R. Hepatic arterial perfusion regulates portal venous flow between hepatic sinusoids and intrahepatic shunts in the normal rat liver in vitro. *Pflugers Arch* 2001; **443**: 257-264

14 **Grisham JW**, Nopanitaya W. Scanning electron microscopy of casts of hepatic microvessels: review of methods and results. In: Lautt WW, ed. Hepatic circulation in health and disease. New York: Raven Press, 1981: 87-109

15 **McCuskey RS**. A dynamic and static study of hepatic arterioles and hepatic sphincters. *Am J Anat* 1966; **119**: 455-477

16 **Bookstein JJ**, Cho KJ, Davis GB, Dail D. Arterioportal communications: observations and hypotheses concerning transsinusoidal and transvasal types. *Radiology* 1982; **142**: 581-590

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Mutations in surface and polymerase gene of chronic hepatitis B patients with coexisting HBsAg and anti-HBs

Hai-Ying Lu, Zheng Zeng, Xiao-Yuan Xu, Nai-Lin Zhang, Min Yu, Wei-Bo Gong

Hai-Ying Lu, Zheng Zeng, Xiao-Yuan Xu, Nai-Lin Zhang, Min Yu, Wei-Bo Gong, Department of Infectious Diseases, Peking University First Hospital, Beijing 100034, China
Supported by the National Natural Science Foundation of China, No. 30271182
Correspondence to: Dr. Zheng Zeng, Department of Infectious Diseases, Peking University First Hospital, Beijing 100034, China. zeng@bjmu.edu.cn
Telephone: +86-10-66551799
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Abstract

AIM: To investigate the clinical significance and presence of mutations in the surface (S) and overlapping polymerase gene of hepatitis B patients with coexisting HBsAg and anti-HBs.

METHODS: Twenty-three patients with chronic hepatitis B were studied. Of the 23 patients, 11 were both positive for hepatitis B virus (HBV) surface antigen (HBsAg) and antibody to HBV surface antigen (anti-HBs), 12 were negative for anti-HBs while positive for HBsAg. DNA was extracted from 200 μL serum of the patients. Nucleotide of the surface and overlapping polymerase gene from HBV-infected patients was amplified by PCR, and the PCR products were sequenced.

RESULTS: Forty-one mutations were found within the surface gene protein of HBV in 15 patients (10 with coexisting HBsAg and anti-HBs). Six (14.6%) out of 41 mutations were located at "α" determinant region in 5 patients (4 positive for HBsAg and anti-HBs). Eleven mutations (26.8%) occurred in the downstream or upstream of "α" determinant region. Lamivudine (LMV)-selected mutations were found in three patients who developed anti-HBs, which occurred in amino acid positions (196, 198, 199) of the surface protein and in YMDD motif (M204I/V) of the polymerase protein simultaneously. Presence of these mutations did not relate to changes in ALT and HBV DNA levels.

CONCLUSION: Besides mutations in the "α" determinant region, mutations at downstream or upstream of the "α" determinant region may contribute to the development of anti-HBs. These mutations do not block the replicating competency of HBV in the presence of high titer of anti-HBs.

INTRODUCTION

Hepatitis B virus (HBV) infection leads to a wide spectrum of liver diseases, including acute self-limited infection, asymptomatic carrier state, fulminant and chronic hepatitis, which could result in life-threatening sequelae, such as liver cirrhosis and hepatocellular carcinoma[1]. In acute self-limited infection, clearance of HBV is associated with seroconversion from HBsAg to anti-HBs due to the coordination of humoral and cellular immune response[2,3]. However, this condition is very rare in chronic infection patients, especially in patients infected at birth, partly because of inadequate humoral and cellular immunity of the host[4]. Furthermore, the prevalence of HBV mutations that could escape from humoral and cellular immunity may result in persistent virus infection.

The S gene of HBV has three open reading frames (ORF), including preS1, preS2 and S region. The surface gene contains a neutralizing epitope named "α" determinant region located at the condon positions 124-147. Mutations in this region could alter the antigenicity of HBsAg, causing failure of anti-HBs to neutralize HBsAg and escaping from the host's immune system, resulting in active viral replication and liver disease[5,6]. It is reported that mutations of some epitopes located at downstream of the "α" determinant region may also affect the neutralization domain[7]. The surface gene overlaps with the calatytic domains of polymerase[7]. Thus, mutations in the surface gene have an effect on the polymerase gene while the polymerase gene mutations also impact the surface gene[8,9].

The present study was to analyze the prevalence of mutations in the surface and polymerase gene of HBV in patients with coexisting anti-HBs and HBsAg.

MATERIALS AND METHODS

Patients

Sera were obtained from 23 Chinese patients with chronic HBV infection. Presence of HBsAg, anti-HBs, hepatitis e antigen (HBeAg), antibody to HBeAg (anti-HBeAg) was detected by commercially available kits according to the instructions. All patients were positive for HBsAg. Of the 23 patients, 11 were positive for anti-HBs (No. 1 to 11) and 20 were positive for HBeAg. Fifteen out of the 23 patients had elevated alanine aminortransferases (ALT) levels. Virological and biochemical parameters of 8 patients positive for anti-HBs and HBsAg at the time of HBV sequence analysis were analyzed. No patients had a history of HBV vaccination or hyper immune globulin therapy. Three patients positive for HBsAg and anti-HBs had a history of lamivudine (LMV) therapy for more than 1 year. All patients were negative for antibody to hepatitis C virus. Sera were stored at -20℃ for DNA extraction.

Primer synthesis

For polymerase chain reaction (PCR), primers were synthesized according to the published sequences. Sequences of the primers for amplifying the surface gene of HBV are as follows: HBV S1: 5'-TTACAGGCGGGGTTTTTC-3' (nt 197, sense); HBV S2: 5'- AAGGGACTCAAGATG-3' -(nt 789, anti-sense). Primers of HBV P1, 5' -GTATTCCCATCCCATCATCC-3' (nt 599, sense) and HBV P2, 5'-CAAGGCAGGATAGCCACATT-3' (nt 1033, anti-sense) were used for amplification of polymerase gene of HBV.

PCR amplification

DNA was extracted from 200 μL serum using a blood DNA kit (Omega, USA). Two microlitres of DNA template and 1 μL of each of the primers, 2 μL of 10 × dNTP and 0.5 U of Taq DNA polymerase (Promega, US) were used in a volume of 50 μL for PCR. After denaturation at 94℃ for 5 min, the reaction for amplification of the surface gene with primers HBV S1 and HBV S2 was carried out at 94℃ for 30 s, at 56℃ for 1 min, and at 72℃ for 1 min for 35 cycles, and a final extension at 72℃ for 10 min. The reaction with primers HBV P1 and HBV P2 for amplifying the polymerase gene was performed at 94℃ for 30 s, at 56℃ for 30 min, at 72℃ for 1 min for 35 cycles, and a final extension at 72℃ for 10 min.

Sequencing of PCR products

The PCR products were purified by centrifugation. Direct sequence of the gene was determined using Taq Dye-Deoxy terminator sequencing kits. Sequencing reactions were analyzed on an automated DNA sequencer (model 377, ABI100, Applied Biosystem). Deduced amino acid sequences were compared with the reported consensus sequence of genotype C, subtype adw, HBV clone (PAK66, PIWK146). Mutations were determined as sequence different from the consensus sequence.

The nucleotide sequence data presented in this paper could be found in the DDBJ/EMBL/GeneBank nucleotide sequence databases with the access numbers AB014381, AB033554, AY812744, AY812743, AY800249, AY123424, AF100309.

Table 1 Clinical data of two groups of patients

Clinical factors	Groups		
	Positive anti-HBs	Negative anti-HBs	*P*
Age in years	40.3 ± 13.2	42.5 ± 14.7	0.7672
Sex, M/F	5/6	7/5	0.6531
Patients with HbsAg, *n* (%)	11 (100)	12 (100)	
Patients with HbeAg, *n* (%)	9 (81.8)	11 (91.7)	
Patients with anti-HBe, *n* (%)	1 (9.1)	0 (0)	
ALT in IU/L	65.9 ± 30.5	98.9 ± 42.0	0.6541
HBV-DNA (log)	7.0 ± 1.60	6.87 ± 0.9	0.5263
Number of amino acid residues mutations in S gene, *n* (%)	34/41 (82.9)	7/41 (17.1)	

Fisher's exact test was used for the categorized data; two-tailed Student's *t* test was used for ALT levels, age and HBV DNA levels (log, copies mL^{-1}).

Statistical analysis

Two-tailed Student's *t* test was used to assess the difference in ALT levels, age, HBV DNA levels between the two groups of patients. Fisher's exact test was used for the analysis of difference in mutations between the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of the clinical features between the two groups of patients is shown in Table 1. There was no significant difference in ALT levels, age, HBV DNA levels between the two groups ($P > 0.05$). The relevant biochemical and virological parameters of 8 patients (No.1 to 6, No.8 and 10) are shown in Table 2.

Nucleotide and deduced amino acid sequences of surface region and polymerase gene of HBV were performed in 23 patients. Comparison with the published HBV sequence showed that 21 (91.3%) out of 23 patients were infected with genotype C, 1 with genotype B and 1 with genotype D.15 (65.2%). Of the 23 patients who developed amino acid mutations in the surface gene protein, 10 were positive for anti-HBs and 5 were negative for anti-HBs. Mutations at the "α" determinant region were observed in 5 patients (5/15, 33.3%) (Figure 1). Forty-one mutations were found at 27 amino acid positions within the surface gene of HBV, and 34 mutations (82.9%, 34/41) were presented in the patients with coexisting HBsAg and anti-HBs. Six (14.6%) out of 41 mutations were located at the "α" determinant region, and 4 mutations were presented in the first loop (positions 124-137), the others were in the second loop (positions 139-147, S143T, G145R). Six mutations at amino acid residues 40 (N40S) and 47 (T47V, T47K, T47R) coincident with HLA class I-restricted (CTL) epitope[10] were observed in 5 patients, 11 mutations (26.8%) occurred in 6 patients within the major hydrophilic regions of upstream and downstream of the "α" determinant region (amino acid positions 99-169), 6 mutations at 3 amino acid positions (196, 198 and 199) associated with LMV-selected

Table 2 Virological and biochemical follow-up data of 8 patients

			2001				2002				2003				2004			
No.	Sex	Age	ALT	HBVDNA	a-HBs	HBeAg	ALT	HBVDNA	a-HBs	HBeAg	ALT	HBVDNA	a-HBs	HBeAg	ALT	HBVDNA	a-HBs	HBeAg
1	M	57	22	0	-	-	20	0	-	-	76	8.63	-	+	87	6.38	+	+
2	M	36	48	9.38	-	+	82	8.53	-	+	21	4.04	-	+	97	3.78	+	+
3	F	65	25	4.04	+	-	58	5.04	+	-	55	6.76	+	-	55	5.08	+	-
4	M	45	45	6.86	+	+	80	6.61	+	+	59	5.57	-	+	93	6.99	+	+
5	M	55	156	7.32	+	+	116	7.11	+	+	112	7.75	+	+	149	5.80	+	+
6	F	30	19	6.91	+	+	33	7.70	+	+	20	8.86	-	+	15	9.08	+	+
8	M	38	18	0	+	-	19	0	+	-	20	0	-	-	21	4.43	+	-
10	M	72	20	5.18	+	+	25	7.04	+	+	18	6.26	-	+	48	4.15	+	+

[+] Positive result; [-] Negative result; ALT: alanine aminotransferase; HBV DNA: HBV DNA levels (log, copies ml^{-1}).

Table 3 Mutations of HBV in polymerase and HBsAg protein

Mutant in patients	Position with HBsAg protein sequence change				Position with polymerase protein sequence change			
(*n*)	145	196	198	199	173	180	204	223
Wild type	G	W	M	W	V	L	M	S
1	-	L	-	-	-	-	I	A
3	-	L	-	-	-	-	I	-
10	-	F	-	C	L	M	V	-
11	R	-	-	-	-	-	-	A
13		L	-	-	-	M	-	-

mutation were observed in 5 patients.

Because the S gene overlaps with the major calatytic domain of the polymerase gene, the mutations near the YMDD motif of the polymerase gene were studied. Eight mutations within amino acid residues 518-569 of the polymerase gene were observed at 4 positions (V173L, L180M, M204I/V, S223A) in 5 patients. Three patients who received long term LMV therapy and developed anti-HBs at the time of sequencing, had YMDD mutations (M204I/V) in polymerase gene and the S gene mutations at amino acid positions 196, 198 and 199 (Table 3).

Five out of 15 (33.3%) patients who had amino acid mutations did not develop anti-HBs, while T131N, L162Q, W196L mutations in the S gene and L180M mutation in polymerase gene were simultaneously observed in only one of these patients.

DISCUSSION

HBV is the most common etiologic agent of chronic and often fetal liver diseases world wide. HBV variants present during natural infection or anti-virus therapy, and contribute to disease persistence. The S gene of HBV is crucial for binding and infectivity, and "α" determinant of the surface gene may form a target of humoral neutralizing antibody. Mutations in the region affect the binding of anti-HBs to corresponding HBsAg[5], produce escape from the neutralizing antibody, result in persistent infection and replication of HBV, even the relatively high titer anti-HBs develops.

In the present study, 15 patients had amino acid mutations in the surface gene of HBV, and 10 of them were positive for anti-HBs. Forty-one mutations were found in 23 patients, and 34 (82.9%) mutations were presented in the patients with coexisting HBsAg and anti-HBs. only 6 mutations within the "α" determinant region were observed in 5 patients (4 for anti-HBs positive). Passive or active immune therapy may develop the escape mutation[11,12], which has a point mutation from guanosine to adenosine at nucleotide 587 (condon 145, G145R). Mutation of G145R, however, was only seen in 1 patient in our study, and it may be the reason why no patient in our cohort received active or passive hepatitis B immunization. The data suggest that mutation within the "α" determinant region may play an important role in the presence of anti-HBs. A resent study showed that mutation in the "α" determinant region contributes to the most therapy failure, but there are still some therapy failures associated with mutations in the major hydrophilic region of the surface gene located at downstream or upstream of the "α" determinant (positions 99-169)[13]. In the present study, 26.8% mutations were observed in the above mention region, suggesting that these mutations also change the antigenicity of HBsAg and contribute to the development of anti-HBs. One patient who had amino acid mutation (T131N) in the "α" determinant region did not develop anti-HBs. It may be due to the relatively lower sensitive assays because it was reported that anti-Hbs complexed with HBsAg could be detected in nearly all patients with chronic hepatitis B when tested by a highly sensitive immunoassay[14].

Lamivudine-selected mutations in the S gene of HBV have been demonstrated by sequencing HBV isolated from the serum of patients treated with long-term LMV[15,16]. In addition, LMV-selected mutations within the HBsAg protein downstream of the "α" determinant (I195M, W196S and M198I) lead to a decrease in the antigenicity of the protein and binding to the anti-HBs antibodies, therefore poorly inhibiting their interaction with wild-type HBsAg[17]. In our study, the change of methionine to isoleucine (rtM204I) or valine (rtM204V) was found in the YMDD motif of the polymerase gene protein in three patients who received long-term LMV therapy. These patients also had W196L/W196F or W199C mutations within the surface gene protein of HBV simultaneously.

The surface gene of HBV also includes the putative

	14	25	40	47	64	76	85	89	95	99	101	110	113	117	122	125	131	134	143	145	159	162	175	196	198
	V	I	N	T	S	C	F	L	L	D	Q/H	L	T	S	K	T	T	F	S	G	A	L	L	W	M
No.				(V)								(I)	(S)		(R)	(M)	(N)		(Y)		(G)	(Q)			
1	G	-	S	V	C	Y	C	I	-	-	-	I	S	-	-	-	-	-	T	-	-	-	-	L	-
2	-	-	-	-	-	-	-	-	-	G	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-
4	-	-	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T
5	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	V	-	-	-	-	-	-	-	I	S	-	R	M	-	Y	-	-	G	-	-	-	-
9	-	F	-	-	-	-	-	-	-	-	R	-	-	-	-	M	-	-	-	-	-	-	S	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-
11	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	Q	-	L	-
13	-	-	-	-	-	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17-23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 1 Amino acid mutations in the surface gene of HBV. Positions of mutation in deduced amino acid residues are indicated by vertical line bellow the surface protein of HBV. The consensus sequences of A, B and D different from those of genotype C are listed in parentheses. Dashes mean residues identical to these reference residues. Patients 1 to 11 were positive for HBsAg and anti-HBs, the others were negative for anti-HBs.

HLA class I-restricted cytotoxic T lymphocyte (CTL) epitopes[2]. Because the putative CTL epitope mutations result in epitope inactivation and T cell receptor antagonism[18], mutant virus could evade cellular immunity and lead to persistent infection[19,20]. In the patients studied here, 6 mutations at amino acid residues 40 and 47 were observed in 1 and 5 patients, respectively, and 5 of them were positive for anti-HBs. These patients had relatively high HBV DNA level. Three of them had elevated ALT levels. This result is similar to the report from Taiwan, which revealed a high frequency of mutations at amino acid positions 40 and 47 of the surface gene in patients with chronic hepatitis B, suggesting that these mutations change CTL recognition and contribute to chronic infection in some patients[10].

In fact, patients positive for anti-HBs have more amino acid mutations, especially mutations in the crucial region of the surface gene associated significantly with the presence of anti-HBs. But presence of these mutations is not related to clinical features, ALT levels, HBV DNA levels and the presence of HBeAg, suggesting that these mutations may not alter the replicating competency of HBV although highly titer of anti-HBs develops. It is possible that these mutants may secrete into the serum via trans-complementation of intact protein in hepatocytes. The biochemical and virological follow-up parameters of 8 patients (No.1 to 6, No.8 and 10) showed that only 2 patients had persistent anti-HBs positive condition during the whole 4 year follow-up period, indicating that anti-HBs can be detected in chronic hepatitis B patients.

In conclusion, the presence of mutations in the "α" determinant of surface gene is not high in patients with coexisting anti-HBs and HBsAg. The mutations at the major hydrophilic region of the surface gene contribute to the development of anti-HBs in these patients and produce escape from the neutralizing antibody, and lead to persistent infection. Long-term LMV therapy could induce YMDD mutation in the polymerase gene and surface gene of HBV.

REFERENCES

1 **Lok AS**. Natural history and control of perinatally acquired hepatitis B virus infection. *Dig Dis* 1992; **10**: 46-52
2 **Chisari FV**, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; **13**: 29-60
3 **Chisari FV**, Ferrari C. Hepatitis B virus immunopathology. *Springer Semin Immunopathol* 1995; **17**: 261-281
4 **Missale G**, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, Rumi MG, Houghton M, Fiaccadori F, Ferrari C**.** Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J Clin Invest* 1996; **98**: 706-714
5 **Zheng X**, Weinberger KM, Gehrke R, Isogawa M, Hilken G, Kemper T, Xu Y, Yang D, Jilg W, Roggendorf M, Lu M. Mutant hepatitis B virus surface antigens (HBsAg) are immunogenic but may have a changed specificity. *Virology* 2004; **329**: 454-464
6 **Swenson PD**, Escobar MR, Carithers RL Jr, Sobieski TJ 3rd. Failure of preexisting antibody against hepatitis B surface antigen to prevent subsequent hepatitis B infection**.** *J Clin Microbiol* 1983; **18**: 305-309
7 **Chen YC**, Delbrook K, Dealwis C, Mimms L, Mushahwar IK, Mandecki W. Discontinuous epitopes of hepatitis B surface antigen derived from a filamentous phage peptide library. *Proc Natl Acad Sci USA* 1996; **93**: 1997-2001
8 **Torresi J**. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol* 2002; **25**: 97-106
9 **Ogura Y**, Kurosaki M, Asahina Y, Enomoto N, Marumo F, Sato C. Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. *J Infect Dis* 1999; **180**: 1444-1451
10 **Tai PC**, Banik D, Lin GI, Pai S, Pai K, Lin MH, Yuoh G, Che S, Hsu SH, Chen TC, Kuo TT, Lee CS, Yang CS, Shih C. Novel and frequent mutations of hepatitis B virus coincide with a major histocompatibility complex class I-restricted T-cell epitope of the surface antigen. *J Virol* 1997; **71**: 4852-4856
11 **Carman WF**, Zanetti AR, Karayiannis P, Waters J, Manzillo G,

Tanzi E, Zuckerman AJ, Thomas HC. Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325-329

12 **Carman WF**, Trautwein C, van Deursen FJ, Colman K, Dornan E, McIntyre G, Waters J, Kliem V, Müller R, Thomas HC, Manns MP. Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis. *Hepatology* 1996; **24**: 489-493

13 **Terrault NA**, Zhou S, McCory RW, Pruett TL, Lake JR, Roberts JP, Ascher NL, Wright TL. Incidence and clinical consequences of surface and polymerase gene mutations in liver transplant recipients on hepatitis B immunoglobulin. *Hepatology* 1998; **28**: 555-561

14 **Maruyama T**, McLachlan A, Iino S, Koike K, Kurokawa K, Milich DR. The serology of chronic hepatitis B infection revisited. *J Clin Invest* 1993; **91**: 2586-2595

15 **Lok AS**, Hussain M, Cursano C, Margotti M, Gramenzi A, Grazi GL, Jovine E, Benardi M, Andreone P. Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e antigen-negative patients receiving lamivudine therapy. *Hepatology* 2000; **32**: 1145-1153

16 **Ogata N**, Fujii K, Takigawa S, Nomoto M, Ichida T, Asakura H. Novel patterns of amino acid mutations in the hepatitis B virus polymerase in association with resistance to lamivudine therapy in japanese patients with chronic hepatitis B. *J Med Virol* 1999; **59**: 270-276

17 **Torresi J,** Earnest-Silveira L, Deliyannis G, Edgtton K, Zhuang H, Locarnini SA, Fyfe J, Sozzi T, Jackson DC. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy**.** *Virology* 2002; **293**: 305-313

18 **Bertoletti A**, Sette A, Chisari FV, Penna A, Levrero M, De Carli M, Fiaccadori F, Ferrari C. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 1994; **369**: 407-410

19 **Aebischer T**, Moskophidis D, Rohrer UH, Zinkernagel RM, Hengartner H. In vitro selection of lymphocytic choriomeningitis virus escape mutants by cytotoxic T lymphocytes. *Proc Natl Acad Sci USA* 1991; **88**: 11047-11051

20 **Pircher H,** Moskophidis D, Rohrer U, Bürki K, Hengartner H, Zinkernagel RM. Viral escape by selection of cytotoxic T cell-resistant virus variants *in vivo*. *Nature* 1990; **346**: 629-633

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PO Box 2345, Beijing 100023, China
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RAPID COMMUNICATION

Pancreatic encephalopathy and Wernicke encephalopathy in association with acute pancreatitis: A clinical study

Guo-Hui Sun, Yun-Sheng Yang, Qing-Sen Liu, Liu-Fang Cheng, Xu-Sheng Huang

Guo-Hui Sun, Yun-Sheng Yang, Qing-Sen Liu, Liu-Fang Cheng, Department of Gastroenterology, Chinese PLA General Hospital, Beijing 100853, China
Xu-Sheng Huang, Department of Neurology, Chinese PLA General Hospital, Beijing 100853, China
Correspondence to: Guo-Hui Sun, Department of Gastroenterology, Chinese PLA General Hospital, Beijing 100853, China. sungh301xhk@yahoo.com.cn
Telephone: +86-10-66937078 Fax: +86-10-68159496
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Abstract

AIM: To investigate clinical characteristics and therapy of pancreatic encephalopathy (PE) and Wernicke encephalopathy (WE).

METHODS: In a retrospective study of 596 patients with acute pancreatitis (AP), patients with PE were compared to those with WE in regards to history, clinical manifestation, diagnosis, treatment and outcome.

RESULTS: There were 93 patients with severe acute pancreatitis (SAP). Encephalopathies were discovered in 10 patients (1.7%). Six patients with PE all developed in SAP (6.5%), and three of them died (3% of SAP, 50% of PE). Four patients with WE developed in AP (0.7%), and two of them died (0.3% of AP, 50% of WE). Two patients with WE were treated with parenteral thiamine and survived. Global confusions were seen in all patients with encephalopathy. Ocular abnormalities were found. Conjugate gaze palsies were seen in 1 of 6 (16.7%) patients with PE. Of 4 patients with WE, one (25%) had conjugate gaze palsies, two (50%) had horizontal nystagmus, three (75%) had diplopia, and one (25%) had myosis. Ataxia was not seen in all patients. None of patients with WE presented with the classic clinical triad. CSF examinations for 2 patients with WE showed lightly-increased proteins and glucose. CT and MRI of the brain had no evidence of characteristic abnormalities.

CONCLUSION: PE occurs in early or reiteration stage of SAP, and WE in restoration stage of SAP/AP. Ocular abnormalities are the hallmarks of WE, and horizontal nystagmus is common. It is difficult to diagnose earlier an encephalopathy as PE or WE, as well as differentiate one from the other. Long fasting, hyperemesis and total parenteral nutrition (TPN) without thiamine are main causes of thiamine deficiency in the course of pancreatitis.

Key words: Pancreatic encephalopathy; Wernicke encephalopathy; Acute pancreatitis; Thiamine; Total parenteral nutrition

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INTRODUCTION

Pancreatic encephalopathy (PE) is an uncommon complication of acute pancreatitis (AP). PE, which is one of multiple organ dysfunction syndrome (MODS), generally occurs in early stage of severe acute pancreatitis (SAP) and has a high mortality of 57%[1]. But in the last or restoration stage of AP, neurological complications are mostly Wernicke encephalopathy (WE) which results from long fasting, hyperemesis and total parenteral nutrition (TPN) without thiamine (vitamin B1, Vit B1). A large dose of Vit B1 is certainly effective for WE. However, it is difficult to diagnose earlier an encephalopathy as PE or WE, as well as differentiate one from the other. Recent studies[2] have shown that WE is poorly recognized by clinicians, even when features of the classic triad of symptoms are evident. Recognition of the progressive nature of the disease is critical because the mortality rate is as high as 10% to 20%[3-5], and treatment may correct all abnormalities. This study demonstrates many of the common clinical characteristics of WE and the diagnostic dilemma physicians encounter when confronted with WE. By reviewing our clinical experience, we can learn many lessons which are beneficial for physicians, not just those who care for patients with PE.

MATERIALS AND METHODS

Patients and methods

A retrospective study was conducted on 596 patients with AP hospitalized at China PLA General Hospital over a 10-year period from Jun 1993 to Dec 2003. There were 93 patients with SAP. A chart was reviewed containing the following demographic and clinical data: age, sex, clinical

Table 1 Manifestation and outcome of patients with encephalopathy

No.	Sex	Age (yr)	Primary disease	Manifestation of encephalopathy	Diagnosis	Treatment/Outcome
1	M	29	SAP with ARDS.	4th d of onset, restlessness, haziness, delirium. Pathological sign negative.	PE	Diazepam, haloperidol. Recovery
2	F	51	SAP	At onset, haziness, delirium.	PE	Recovery
3	M	41	SAP with ALI.	4th d of onset, restlessness, sleepiness, haziness.	PE	Recovery
4	M	34	SAP with pseudocyst bleeding	SAP for 3 mo, 3rd d after pseudocyst operation, hallucination, delirium, conjugate gaze palsies, coma, suspected Kernig sign. Diffused pancreatic necroses.	PE	Death
5	F	42	SAP with ALI and shock	9th d of onset, delirium, unconsciousness.	PE	Death
6	M	31	SAP with ARF and ARDS.	33rd d after onset, restlessness, hebetude, unconsciousness, delirium.	PE	Death
7	F	37	Recovery Phase of AP	Protracted vomiting. No supplement of $VitB_1$. 36th d after onset, diplopia, tinnitus, apathy, dizziness, horizontal nystagmus. CSF negative, MRI negative.	WE	$VitB_1$, B_{12} im. Recovery after 4 d
8	M	48	Recovery Phase of SAP, with ALI and pseudocyst	45th d of onset, diplopia, sleepiness, haziness, horizontal nystagmus, spatial disorientation, decreased tendon reflex.	WE. Once suspected PE	Fasting for 51 d, no $VitB_1$ in TPN for 44 d. Recovery after 4-day's administration of $VitB_1$
9	M	37	Acute recurrent pancreatitis with pseudocyst	Long fasting, no supplement of $VitB_1$. Nausea, vomiting, dizziness, hypomnesia, alalia, diplopia, amentia, coma. Conjugate gaze palsies, active tendon reflex, ankle clonus positive. CSF: total cells 134, WBC 2; glucose, protein increased lightly. MRI: suspected focus of brain stem.	WE. Once suspected PE and encephalitis	Dexamethasone ineffective. Administration of $VitB_1$, 400 mg/d. Death
10	F	40	Recovery phase of AP	42nd d after onset, vomiting, dizziness, alalia, trance, amentia, sleeplessness, hyperspasmia, coma. Myosis, decreased tendon reflex. Pathological signs negative. CSF: protein positive; total cells 180, WBC 0; glucose, protein increased lightly.	WE. Once suspected viral encephalitis	Dexamethasone, acyclovir ineffective. Respirator. Death

ALI: acute lung injury; ARF: acute renal failure; ARDS: acute respiratory distress syndrome; CSF: cerebrospinal fluid; TPN: total parenteral nutrition.

signs, history, imaging, treatment, hospital course and outcome. Patients with PE were then compared to those with WE in regards to history, clinical manifestation, diagnosis, treatment, and outcome.

RESULTS

Encephalopathy was discovered in 10 patients (1.7%), including 6 males and 4 females with a mean age of 39.0 years (from 29 to 51 years old). Six patients with PE all developed from SAP (6.5%); three patients died (3% of SAP, 50% of PE). Four patients with WE developed from AP (0.7%); two patients died (0.3% of AP, 50% of WE). Two patients with WE were treated with parenteral Vit B1 and survived. PE occurred in early or reiteration stage of SAP, and WE in restoration stage of SAP/AP.

The clinical features were also reviewed in all patients (Table 1). Global confusions were seen in all patients with encephalopathy. Ocular abnormalities were also found. Conjugate gaze palsies were seen in 1 of 6 (16.7%) patients with PE. Of 4 patients with WE, 1 (25%) was seen conjugate gaze palsies, 2 (50%) horizontal nystagmus, 3 (75%) diplopia, and 1 (25%) myosis. Ataxia was not seen in any of the patients. None of the patients with WE presented with the classic clinical triad. CSF examination was performed on 1 patient with PE and showed negative, whereas it was done on 2 patients with WE and showed lightly-increased proteins and glucose. Of the 10 patients, 5 had CT scan of the brain and none had evidence of characteristic abnormalities. MRI was performed on 2 patients with WE and only 1 had some suspected changes of brain stem. Long fasting, protracted vomiting and TPN without Vit B1 supplement were main causes of Vit B1 deficiency, which crucially resulted in WE.

DISCUSSION

PE, first described by Lowell in 1923, refers to the abnormalities of mental status in patients with AP. Abnormalities of mental status, such as spatial disorientation, trance, agitation with delusion and hallucination, were defined as PE by Rothermich[6,7]. In China, PE had a high mortality of 57%[1], and main causes of death were shock, MODS, renal failure and ketoacidosis. At present pathogenesis of PE is unclear yet, and most scholars think that it is related to phospholipase A (PLA) activation, hypovolemia, multiple organ failure, electrolyte disturbance and cytokine effect in the course of AP[8]. In recent years, it has been gradually known that PLA in pancreatitis is not only the primary factor causing pancreatic necrosis, but also crucial substance resulting in PE[9]. Johnson *et al*[10] reported a male patient with AP. Cerebral fat embolism was established as the cause of his death. They thought that PE might be due to hypoxia secondary to pulmonary fat embolism, cerebral fat embolism, or the complicating syndromes of disseminated intravascular coagulation or hyperosmolality.

PLA2 damages structural phospholipid of brain cell membrane; platelet activating factor (PAF) increases intracerebral vascular permeability with brain edema and demyelinization of grey and white matter. PE is primarily due to the demyelinization of the cerebral grey and white matter caused by PLA2, which can induce increased vascular permeability. The intravascular osmotic pressure decreases and the brain becomes more vulnerable to transudation, and finally brain edema is resulted[11].

With proper treatment, the recovery in patients aged

below 40 is uneventful. Those older than 60 especially those with a previous history of cerebral infarction may have some sequela[11]. Ruggieri *et al*[12] presented a patient of 43-year-old man who, after an acute episode of pancreatitis, experienced five relapses, with alternating focal signs. The patient had improved, but cognitive impairment persisted after a 7-year follow-up.

A report[13] of Boon *et al* showed the usefulness of MRI in the diagnosis of this disorder. Patchy white matter signal abnormalities, resembling plaques seen in multiple sclerosis, might reflect the lesions that were found in the cerebral white matter of post-mortem confirmed patients.

Estrada *et al*[7] conducted a prospective study on 17 patients with AP. PE was discovered in 6 patients (35%). A direct relationship was found to exist between the PE condition and an increase in CSF-lipase, and electroencephalographic changes were nonspecific. The encephalopathy did not affect the course of AP, and showed no relationship to type of treatments involved. Whereas the severity of AP was not related to the presence or absence of encephalopathy.

Our study revealed that PE occurred in early or reiteration stage of SAP and had a global confusional state for 2-7 d. Some patients had ocular abnormalities. There was no specific therapy for PE. Treatment of AP was the key to prevention and therapy of PE.

The clinical trial of Qian *et al*[14] revealed that recombinant human growth hormone (rhGH) had a therapeutic effect for patients with early PE; rhGH combined with somatostatin might reduce occurrence of PE. However, the mechanism is unclear yet.

WE is an uncommon neurological disorder characterized by a triad of ocular abnormalities, ataxia, and global confusional state. In 1881, Carl Wernicke initially described punctate hemorrhages affecting grey matter around the third and fourth ventricle and aqueduct of Sylvius and designated it "polioencephalitis hemorrhagica superioris"[15]. Experimental and clinical studies have demonstrated that WE results from a deficiency of thiamine (vit B1), an essential coenzyme in intermediate carbohydrate metabolism[16,17].

The mortality rate ranges from 10% to 20%. At autopsy, patients may have pin-point hemorrhages in the mamillary bodies, hypothalamus, and paraventricular regions of the thalamus, around the aqueduct and beneath the floor of the fourth ventricle[3-5]. A retrospective study of Ogershok *et al* showed similar pathological lesions at autopsy[2].

Although WE is thought to be a disease that occurs primarily in the alcoholic population, Lindboe's autopsy study revealed 12 (23%) of 52 patients in nonalcoholic population[4]. Some of the nonalcoholic conditions associated with this disorder include prolonged intravenous feeding, hyperemesis gravidarum, anorexia nervosa, refeeding after starvation, thyrotoxicosis, regional enteritis, malabsorption syndromes, hemodialysis, peritoneal dialysis, uremia, HIV, malignancy, and gastroplasty with postoperative vomiting[15,18,19].

WE is a life-threatening condition that is avoidable by early recognition and administration of thiamine. Recognition of this disorder remains difficult because very few patients actually present with the classic signs of nystagmus, ataxia, and global confusion. To deal with this diagnostic problem for chronic alcoholics, new operational criteria were published in 1997. The diagnosis criteria require 2 of the following 4 signs: dietary deficiencies, oculomotor abnormalities, cerebellar dysfunction, and either an altered mental state or mild memory impairment[20]. Harper *et al*[3] indicated that only about 16% had this classic triad, and 19% had no clinical signs. On Harper's review of both clinical and pathology studies, a wide range of the presentation with these signs was revealed. Ocular signs were present in 29% to 93%, ataxia in 23% to 70%, and mental changes in 82% to 90%.

In fact, none of the CT studies helps clinicians with the diagnosis. MRI can reveal areas of signal change in the paraventricular regions of the thalamus and in the paraventricular regions of the midbrain with this disorder. Dilation of the third ventricle and atrophy of the mamillary bodies can also be seen. MRI is helpful in confirming the diagnosis of WE; however, the absence of abnormalities does not exclude the diagnosis. The sensitivity of MRI is 53%, whereas the specificity is 93%[21-24].

WE is a medical emergency and treatment consists of hospital admission and administration of intravenous thiamine. The clinical response after administering thiamine is usually striking and rapid enough to be virtually diagnostic[3]. As few as 2 mg of thiamine may be enough to reverse the ocular symptoms (which generally begin to improve in 1-6 h), however, initial doses of at least 100 mg are usually administered. Ataxia and acute confusional state may resolve dramatically, although improvement may not be noted for days or months. We suggest starting thiamine prior to treatment with IV glucose solutions, and continuing until the patient resumes a normal diet. Magnesium is an indispensable cofactor in thiamine-dependent metabolism. In hypomagnesemic states, normal function of thiamine pyrophosphate, the active coenzyme containing thiamine, does not occur. Consequently, the final step in treating WE is correcting magnesium deficiency. The prognosis of WE depends on the stage of disease and prompt institution of thiamine[15,16].

Our study revealed that WE continued to be a rare but life-threatening condition often overlooked in the course of AP. WE occurred in restoration stage of SAP/AP. Ocular abnormalities were the hallmarks of WE, and horizontal nystagmus was common. Long fasting, hyperemesis and TPN without Vit B1 in the course of AP were main causes of Vit B1 deficiency. Two patients with WE were treated with parenteral Vit B1 and survived; two patients once misdiagnosed as PE or cephalitis.

Chen *et al*[1] analyzed 185 patients with AP complicated with encephalopathy. They thought that encephalopathy appearing in the early course of AP was PE. PE had a high mortality of 57%. WE appeared in the late course of AP (> 2 wk or in recovery period), and had a mortality of 33% (26/78). The difference between the two groups was significant ($P < 0.01$). Supplement of thiamine in time resulted in lower mortality in WE. Therefore, they suggested that patients who have been on fasting for a long time (more than 10 d) should be given thiamine intramuscularly in case WE occurs.

Winslet *et al*[25] reported a young obese female with AP complicated by pseudocyst formation and intermittent

gastric outlet obstruction, who had been maintained on high-calorie enteral feeds, developed a sudden onset of confusion and ophthalmoplegia associated with papilloedema and retinal haemorrhages. A possible diagnosis of WE was made. The patient was treated with parenteral thiamine, and survived. The authors suggested that any patient with suspicious or unusual neurological symptoms and signs associated with possible malnutrition, hyperemesis or malabsorption should be given intravenous thiamine without delay to avoid the potential morbidity and mortality associated with undiagnosed WE.

In summary, it is difficult to diagnose earlier PE and WE complicating AP. In case differential diagnosis of PE and WE is baffled, Vit B1 diagnostic treatment may be useful: patients' condition of WE is supposed to improve after injected Vit B1 (100 mg/d) therapy for 1-3 d. If a patient, in the course of pancreatitis, has suspicious or unusual neurological symptoms and signs, a possible diagnosis of encephalopathy should be made, and the patient should be given intravenous thiamine without delay to avoid the potential morbidity and mortality associated with undiagnosed WE.

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REFERENCES

1 **Chen L**, Zhang X. [Pancreatic encephalopathy and Wernicke encephalopathy]. *Zhonghua Neike Zazhi* 2002; **41**: 94-97
2 **Ogershok PR**, Rahman A, Nestor S, Brick J. Wernicke encephalopathy in nonalcoholic patients. *Am J Med Sci* 2002; **323**: 107-111
3 **Harper CG**, Giles M, Finlay-Jones R. Clinical signs in the Wernicke-Korsakoff complex: a retrospective analysis of 131 cases diagnosed at necropsy. *J Neurol Neurosurg Psychiatry* 1986; **49**: 341-345
4 **Lindboe CF**, Løberg EM. Wernicke's encephalopathy in nonalcoholics. An autopsy study. *J Neurol Sci* 1989; **90**: 125-129
5 **Harper C**, Gold J, Rodriguez M, Perdices M. The prevalence of the Wernicke-Korsakoff syndrome in Sydney, Australia: a prospective necropsy study. *J Neurol Neurosurg Psychiatry* 1989; **52**: 282-285
6 **Menza MA**, Murray GB. Pancreatic encephalopathy. *Biol Psychiatry* 1989; **25**: 781-784
7 **Estrada RV**, Moreno J, Martinez E, Hernandez MC, Gilsanz G, Gilsanz V. Pancreatic encephalopathy. *Acta Neurol Scand* 1979; **59**: 135-139
8 **Pitchumoni CS**, Agarwal N, Jain NK. Systemic complications of acute pancreatitis. *Am J Gastroenterol* 1988; **83**: 597-606
9 **Nevalainen TJ**, Hietaranta AJ, Gronroos JM. Phospholipase A2 in acute pancreatitis: new biochemical and pathological aspects. *Hepatogastroenterology* 1999; **46**: 2731-2735
10 **Johnson DA**, Tong NT. Pancreatic encephalopathy. *South Med J* 1977; **70**: 165-167
11 **Wu XN**. Current concept of pathogenesis of severe acute pancreatitis. *World J Gastroenterol* 2000; **6**: 32-36
12 **Ruggieri RM**, Lupo I, Piccoli F. Pancreatic encephalopathy: a 7-year follow-up case report and review of the literature. *Neurol Sci* 2002; **23**: 203-205
13 **Boon P**, de Reuck J, Achten E, de Bleecker J. Pancreatic encephalopathy. A case report and review of the literature. *Clin Neurol Neurosurg* 1991; **93**: 137-141
14 **Qian Zhu-Yin**, Miao Yi, Liu Xun-Liang, Du Jing-Hui. Clinical experience on the therapy of pancreatic encephalopathy. *Zhongguo Xiandai Putong Waike Jinzhan* 2001; **4**: 99-100
15 **Zubaran C**, Fernandes JG, Rodnight R. Wernicke-Korsakoff syndrome. *Postgrad Med J* 1997; **73**: 27-31
16 **Reuler JB**, Girard DE, Cooney TG. Current concepts. Wernicke's encephalopathy. *N Engl J Med* 1985; **312**: 1035-1039
17 **Davis RE**, Icke GC. Clinical chemistry of thiamin. *Adv Clin Chem* 1983; **23**: 93-140
18 **Harper CG**. Sudden, unexpected death and Wernicke's encephalopathy: a complication of prolonged intravenous feeding. *Aust N Z J Med* 1980; **10**: 230-235
19 **Parkin AJ**, Blunden J, Rees JE, Hunkin NM. Wernicke-Korsakoff syndrome of nonalcoholic origin. *Brain Cogn* 1991; **15**: 69-82
20 **Caine D**, Halliday GM, Kril JJ, Harper CG. Operational criteria for the classification of chronic alcoholics: identification of Wernicke's encephalopathy. *J Neurol Neurosurg Psychiatry* 1997; **62**: 51-60
21 **Suzuki S**, Ichijo M, Fujii H, Matsuoka Y, Ogawa Y. Acute Wernicke's encephalopathy: comparison of magnetic resonance images and autopsy findings. *Intern Med* 1996; **35**: 831-834
22 **Yokote K**, Miyagi K, Kuzuhara S, Yamanouchi H, Yamada H. Wernicke encephalopathy: follow-up study by CT and MR. *J Comput Assist Tomogr* 1991; **15**: 835-838
23 **Antunez E**, Estruch R, Cardenal C, Nicolas JM, Fernandez-Sola J, Urbano-Marquez A. Usefulness of CT and MR imaging in the diagnosis of acute Wernicke's encephalopathy. *AJR Am J Roentgenol* 1998; **171**: 1131-1137
24 **Shin RK**, Galetta SL, Imbesi SG. Wernicke encephalopathy. *Arch Neurol* 2000; **57**: 405
25 **Winslet MC**, Donovon IA, Aitchison F. Wernicke's encephalopathy in association with complicated acute pancreatitis and morbid obesity. *Br J Clin Pract* 1990; **44**: 771-773

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Disordered beta-catenin expression and E-cadherin/CDH1 promoter methylation in gastric carcinoma

Li Wang, Fan Zhang, Ping-Ping Wu, Xu-Cheng Jiang, Lin Zheng, Ying-Yan Yu

Li Wang, Fan Zhang, Ping-Ping Wu, Xu-Cheng Jiang, Lin Zheng, Ying-Yan Yu, Department of Pathology, School of Medicine, Shanghai Jiaotong University, Shanghai 200025, China
Supported by Shanghai Higher Education Science & Technology Funds, No. 02BK07, and Shanghai Science Development Funds, No. 02ZB14047
Correspondence to: Xu-Cheng Jiang, Department of Pathology, School of Medicine, Shanghai Jiaotong University, Shanghai 200025, China. xjiang@shsmu.edu.cn
Telephone: +86-21-63846590-776420
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Abstract

AIM: To investigate the distribution of beta-catenin in nuclei or membrane/cytoplasm of gastric carcinoma cells, the relationship between E-cadherin gene methylation and its expression, and the role of beta-catenin and E-cadherin as potential molecular markers in predicting tumor infiltration.

METHODS: Twenty-nine cases of gastric carcinoma, classified as diffuse and intestinal variants, were selected for study. Nuclear and cytoplasmic proteins were purified and beta-catenin content was detected by ELISA. DNA methylation of E-cadherin/CDH1 gene promoter was studied by methylation-specific PCR and compaired with E-cadherin expression detected by immunohistochemistry.

RESULTS: In 27 cases of gastric carcinoma, the ratio of beta-catenin content between nuclei and membrane/cytoplasm was correlated with the T-classification (r = 0.392, P = 0.043). The significance was present between T2 and T3 groups. No correlation was detected between diffuse and intestinal variants in terms of their beta-catenin distribution. In 21 cases of diffuse variants of gastric carcinoma, there was a difference in E-cadherin expression between CDH1 gene-methylated group and non-methylated group (29 % *vs* 71 %, P = 0.027). No correlation between CDH1 gene methylation and T-classification was found, neither was the significance between E-cadherin expression and tumor infiltration grade.

CONCLUSION: Comparative analysis of nuclear and membrane/cytoplasmic beta-catenin can predict local tumor infiltration. E-cadherin/CDH1 gene methylation is an important cause for its gene silence in diffuse variant gastric carcinoma. Methylation of CDH1 gene in the absence of E-cadherin is an early event in gastric carcinogenesis.

Key words: Gastric carcinoma; Beta-catenin; E-cadherin; DNA methylation

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INTRODUCTION

Gastric carcinoma is highly malignant and usually results in a poor outcome. Until now there is no satisfactory tumor marker for predicting its evolution. E-cadherin, a transmembrane glycoprotein of 120 KD which is expressed in normal epithelium, may play an important role both in cell-cell adhesion and in tumor invasion and metastasis. The low expression of E-cadherin may favor the dissociation of carcinoma cells from one another for their invading out of basal membrane. Though mutation and allelic loss have been confirmed as major mechanisms for E-cadherin (CDH1) gene inactivation in many malignancies[1], it has been recently reported that CDH1 promoter methylation could be frequently detected in gastric carcinoma[2]. Still it is controversial whether DNA methylation is the main cause for E-cadherin/CDH1 gene silence. As a copartner of E-cadherin, beta-catenin is critical for intercellular adhesion in membrane and cytoplasm of cells, it also plays a role as a transcription activating protein in nuclei[3]. The nuclear accumulation of beta-catenin may stimulate gastric epithelium proliferation[4], nevertheless the effect of beta-catenin on tumor infiltration in gastric carcinoma is waiting to be more precisely studied by quantitative analysis. The aim of the present study was to investigate the relationship between E-cadherin gene methylation and its expression, the distribution of beta-catenin in nuclei and cytoplasm in gastric carcinoma, and the role of beta-catenin and E-cadherin as potential molecular markers in predicting tumor infiltration.

MATERIALS AND METHODS

Patients and tumor samples

Tissue blocks were obtained from the Department of Digestive Disease, Shanghai Ruijing Hospital and the Department of Pathology, Shanghai No.2 Hospital, involving 29 cases of gastric carcinoma operated from 2002 to 2003, of which 21 were diffuse variants and 8 were intestinal variants, together with 5 paraneoplastic non-tumor gastric tissues. The samples were freshly frozen at -70℃ for DNA and protein extraction. Also the samples were fixed in 40 g/L formalin buffer then paraffin-embedded routinely for immunohistochemistry. T-classification revealed that 3 cases were T1, 5 T2, 18 T3 and 3 T4.

ELISA for nuclear/cytoplasmic beta-catenin

Nuclear and cytoplasmic protein was isolated for beta-catenin analysis. Frozen tumor tissue (1.0 cm × 1.0 cm × 1.0 cm) from each case was cut into minimal sections and homogenized manually for 5 min at 4℃ with 700 μL cytoplasmic lysis buffer (0.15 mol/L NaCl, 10 mmol/L HEPES, 1mmol/L EDTA, 6 mL/L NP-40). Membrane and cytoplasmic lysis were checked by microscopic examination. The nuclei were collected by centrifuging for 5 min at 1 300 r/min at 4℃, then vigorously homogenized for 30 min at 4℃ with 500 μL nuclear lysis buffer (0.4 mol/L NaCl, 20 mmol/L HEPES, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, 250 mL/L glycerol, 1.2 mmol/L MgCl2, 0.5 mmol/L DTT, 0.5 mg/L leupeptin, 0.5 mg/L aprotinin, 0.5 mg/L pepstatin). The protein in nuclear or cytoplasmic solution was tested by Coomassie reagents following the manufacturer's instructions (Coomassie Plus-200 protein assay reagent, No-23238, Hyclone-PIERCE, USA).

Nuclear or cytoplasmic content of beta-catenin was analyzed by ELISA. The proteins were immobilized onto 96-well microtiter plates at 4℃, and washed with PBS-0.5 mL/L Tween 20. Monoclonal mouse antibody (anti-beta-catenin, M-0545, Antibody Diagnostica Company, USA) was applied in 1:25 dilution of PBS-0.5 mL/L Tween 20 at 37℃ for 1h. After being washed, the wells were incubated with AKP-conjugated secondary antibody and then washed again and 1mg/mL pNPP was added. Absorbance of eluted dye was measured at A312nm. Negative controls were performed by replacing primary antibody with PBS-0.5 mL/L Tween 20.

Methylation-specific PCR (MSP) for E-cadherin/CDH1 gene

For each sample, the frozen gastric tissue (0.5 cm × 0.5 cm × 0.5 cm) was cut into minimal sections and incubated with proteinase K (20 g/L) at 55℃ for 24 h. The DNA was extracted by standard phenol/chloroform technique.

DNA samples (100 μL) were incubated in 0.2 mol/L NaOH at 37℃ for 10 min, then modified with sodium bisulfite solution following the manufacturer's instructions (CpGenome DNA modification kit, S7820, Ingergen Company). The modified DNA was amplified with E-cadherin/CDH1 gene-specific primers as follow: methylated-specific primer set: sense 5'-GGTGAAT TTTTAGTTAATTAGCCGGTAC-3' and antisense 5'-CATAACTAACCG AAAACGCCG-3', yielding a product of 204 bp; unmethylated-specific primer set: sense 5'-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3' and antisense 5'-ACCCATAACTAACCAAAAACACCA-3', yielding a product of 211 bp[5]. The PCR mixture (50 μL in total) contained 1 × buffer (SABC Biochemical) with 1.5 mmol/L $MgCl_2$, 0.2 mmol dNTPs, 0.2 μmol of each primer, and 4 μL of DNA sample. PCR conditions were 10 min at 94℃, after which 3U of Taq DNA polymerase (SABC Biochemical) was added, and 35 cycles at 94℃ for 50 s, at 57℃ for 40 s, at 72℃ for 90 s, and a final extension at 72℃ for 5 min. The positive control was performed on DNA from normal gastric tissue by using unmethylated-specific primers, and the negative control was prepared on PCR mixture without primers. The PCR products were migrated by electrophoresis on 20 g/L agarose gel, with 100 bp DNA ladder as a DNA marker.

Immunohistochemistry (IHC) for E-cadherin expression

Formalin-fixed paraffin-embedded tissue sections (4 μm thick) were deparaffinized in xylene for 10 min, and rehydrated through graded alcohols to water. Antigen retrieval was performed by microwave of tissue sections in 10 mmol/L sodium citrate buffer (pH 6.0) for 15 min at 750 W. Endogenous peroxidase activity was blocked with 3 mL/L hydrogen peroxide. Primary antibody (anti-E-cadherin, mouse monoclonal antibody, Antibody Diagnostic Company, USA) was applied at 1:25 dilution and incubated for 1 h at room temperature. The slides were washed in PBS for 15 min, and secondary incubations were carried out by using anti-mouse antibody-polymerized dextran-HRP complex (ADI Two-Step System, Antibody Diagnostica Company, USA) for 30 min. Immunoreactivity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin. The brown-stained color in cell membrane by DAB was defined as positive reactivity. Negative controls were performed by replacing primary antibody with PBS. The DAB staining in nuclei or cytoplasm was considered as negative.

The expression of E-cadherin was considered as positive when at least 10% of tumor cells were colored by DAB[6]. In quantitative evaluation, 5 microscopic fields were analyzed on each tissue slide. The percentage of positive cells (300 to 900 tumor cells counted for each sample) was classified as grade 1 (11%-25%), grade 2 (26%-50%), grade 3 (51%-75%) and grade 4 (more than 75%).

Statistical analysis

Fisher's exact probability test was used to evaluate the relation between E-cadherin/CDH1 gene methylation and its expression. Correlations among the E-cadherin protein, the ratio of beta-catenin content between nuclei and membrane/cytoplasm, the tumor infiltration grading and the Lauren's typing were evaluated by method of Spearman (statistical software: SAS 6.12).

RESULTS

ELISA for nuclear/cytoplasmic beta-catenin

Twenty-seven cases of gastric carcinoma were successfully analyzed for beta-catenin distribution. The ratio of beta-catenin content between nuclei and membrane/cytoplasm

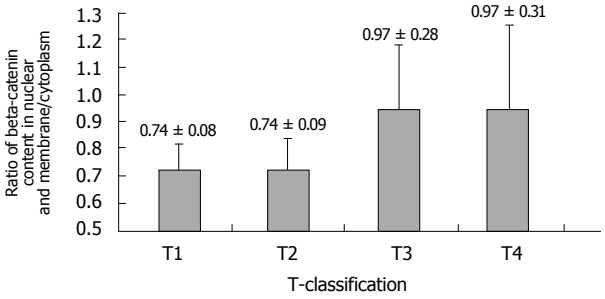

Figure 1 Ratio of beta-catenin content between nuclei and membrane/cytoplasm in different groups of gastric carcinoma according to the T-classification levels.

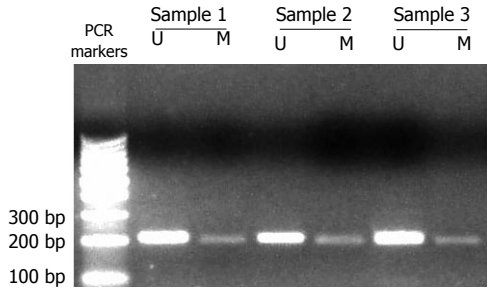

Figure 2 Methylation-specific PCR (MSP) analysis of E-cadherin/CDH1 gene in gastric carcinoma. Sodium bisulfite-modified DNA samples were subjected to PCR with specific primers. Existence of methylation was demonstrated by the presence of PCR product (204 bp) rendered by methylated specific primers. M: methylated-specific primer sets; U: unmethylated-specific primer sets.

was correlated with the T-classification (r = 0.392, P = 0.043). The significance was present between T2 and T3 groups (Figure 1). There was no relation between diffuse variant and intestinal variant in terms of their beta-catenin distribution in tumor cells.

Methylation-specific PCR for E-cadherin/CDH1 gene

In 29 cases of gastric carcinoma, CDH1 gene promoter methylation was identified in 13 cases (45%) (Figure 2), of which 7 cases were histologically diffuse variants (33%), 6 cases intestinal variants (75%). All 3 T1 cases were found to be positive for CDH1 gene methylation, so did 1 of 5 T2 cases, 7 of 18 T3 cases and 2 of 3 T4 cases. There was no difference in diffuse and intestinal variants for their CDH1 gene methylation, and no correlation between CDH1 gene methylation and T-classification was found. The CDH1 gene methylation was also identified in 3 specimens of paraneoplastic non-tumor gastric tissues.

Immunohistochemistry for E-cadherin expression

E-cadherin expression was detected in 18 of 29 gastric carcinoma cases (62%) (Figure 3), of them 12 cases were diffuse variants (57%) and 6 cases intestinal variants (75%). Two of 3 T1 cases, 2 of 5 T2 cases, 11 of 18 T3 cases and all 3 T4 cases were found to be positive for E-cadherin expression.

Of the 13 gastric carcinoma cases which were identified for CDH1 gene methylation, E-cadherin expression was found in 2 of 7 diffuse variants and 5 of 6 intestinal variants. In 21 cases of diffuse variants, there was a difference in E-cadherin expression between CDH1 gene-methylated group and non-methylated group (29% *vs* 71%, P = 0.027). No significance between E-cadherin expression and T-classification was found, neither was the relation between diffuse variant and intestinal variant for their E-cadherin expression.

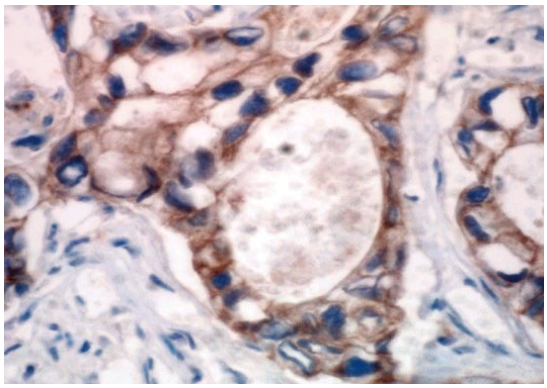

Figure 3 Immunohistochemical analysis of E-cadherin expression in gastric carcinoma (DAB × 400). Positive reaction was assessed as membrane reactivity.

DISCUSSION

Our previous study demonstrated that E-cadherin and beta-catenin are co-expressed in gastric carcinoma[7]. The regulation at their transcriptional levels seems to be independently controlled by different mechanisms[8]. Beta-catenin is a bifunctional protein, location in nuclear or cytoplasm/membrane is critical for its activity as an adhesive factor or proto-oncoprotein. In nuclei, beta-catenin plays a role as a target of the wnt signaling pathway in stimulating cell proliferation through activation of cyclin D1[9] and c-myc gene[10]. The nuclear translocation of beta-catenin depends on its stabilization and the P13K signaling pathway. The latter may be activated by EB virus infection[11], which is also thought to be an important cause for E-cadherin/CDH1 gene methylation[12].

The nuclear accumulation of beta-catenin seems to be helpful, regarding tumor infiltration in gastric[13] and colorectal carcinoma[14], but this accumulation could also be found in early neoplasia or even in non-tumor metaplasia[4], and beta-catenin expression at cell level does not appear to change from normal to carcinoma epithelium[15]. Our results indicated that the evaluation of both nuclear and membrane/cytoplasmic beta-catenin expression might have a prognostic implication and a potential diagnostic value for gastric carcinoma. In the present study, we found a significant variation of beta-catenin distribution from T2 to T3 grade, suggesting that beta-catenin plays an important role in tumor infiltration in gastric smooth muscle. The possible mechanism may be that beta-catenin could regulate the matrix metalloproteinase activity in degradation of extracellular matrix components[16], and this regulation is mainly focused on local infiltration rather than metastasis[17].

E-cadherin is an indispensable protein for cell adhesion in normal epithelium. Loss of its function is propitious to tumor invasion. For silence of the E-cadherin/CDH1 gene in gastric carcinoma, recent studies showed that not only the changes of DNA sequence but also the epigenetic

modification[18,19] such as DNA methylation, may play an important role in the loss of E-cadherin expression.

DNA methylation has attracted great attention recently, as many tumor suppresser genes contain CpG-rich promoters called CpG islands, which are normally unmethylated. Silence of these genes may occur in association with the aberrant hypermethylation of their promoter regions. It has been reported that E-cadherin/CDH1 gene is frequently methylated in colorectal and gastric carcinoma, but its endogenetic changes such as mutation are rare[12,20]. An *in vitro* study demonstrated that the E-cadherin expression, associated with the cell metamorphosis, could be induced by demethylation drugs[21]. These studies indicate that DNA methylation may be a possible mechanism for E-cadherin/CDH1 gene silence in human gastric carcinoma.

In the present study, 45% of gastric carcinomas were positive for E-cadherin/CDH1 gene methylation, which corresponds to the result of previous study[22]. We showed that E-cadherin expression was negatively correlated with its gene methylation in diffuse variants, indicating that DNA methylation may diminish E-cadherin expression and thus favor the devastating activity of the tumor. The data reported here do not provide evidence of correlation between CDH1 gene methylation and tumor infiltration, but are in agreement with the hypothesis that methylation status of CDH1 promoter may be an early event in gastric carcinogenesis[15].

In conclusion, comparative analysis of nuclear and membrane/cytoplasmic beta-catenin can predict tumor infiltration in gastric wall, and the E-cadherin/CDH1 gene methylation is an important cause for its gene silence in diffuse variant gastric carcinomas. Analysis for detecting distribution of beta-catenin protein and methylation state of E-cadherin/CDH1 gene helps to understand the dynamic process of oncogenesis.

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REFERENCES

1 **Oda T**, Kanai Y, Oyama T, Yoshiura K, Shimoyama Y, Birchmeier W, Sugimura T, Hirohashi S. E-cadherin gene mutations in human gastric carcinoma cell lines. *Proc Natl Acad Sci USA* 1994; **91**: 1858-1862

2 **Graziano F**, Arduini F, Ruzzo A, Mandolesi A, Bearzi I, Silva R, Muretto P, Testa E, Mari D, Magnani M, Scartozzi M, Cascinu S. Combined analysis of E-cadherin gene (CDH1) promoter hypermethylation and E-cadherin protein expression in patients with gastric cancer: implications for treatment with demethylating drugs. *Ann Oncol* 2004; **15**: 489-492

3 **Cadigan KM**, Nusse R. Wnt signaling: a common theme in animal development. *Genes Dev* 1997; **11**: 3286-3305

4 **Romiti A**, Zullo A, Borrini F, Sarcina I, Hassan C, Winn S, Tomao S, Vecchione A, Morini S, Mingazzini P. Relationship between beta-catenin expression and epithelial cell proliferation in gastric mucosa with intestinal metaplasia. *World J Gastroenterol* 2005; **11**: 4400-4403

5 **Graff JR**, Herman JG, Myöhänen S, Baylin SB, Vertino PM. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. *J Biol Chem* 1997; **272**: 22322-22329

6 **Shiozaki H**, Iihara K, Oka H, Kadowaki T, Matsui S, Gofuku J, Inoue M, Nagafuchi A, Tsukita S, Mori T. Immunohistochemical detection of alpha-catenin expression in human cancers. *Am J Pathol* 1994; **144**: 667-674

7 **Wang L**, Zhang F, Wu PP, Jiang XC, Zheng L, Yu YY. Relationgship between E-cadherin or β-catenin Expression and Gastric Carcinoma. *J Shanghai Sec Medi Univ* 2005; **25**: 675-678

8 **Lynch HT**, Grady W, Suriano G, Huntsman D. Gastric cancer: new genetic developments. *J Surg Oncol* 2005; **90**: 114 -133; discussion 133

9 **Utsunomiya T**, Doki Y, Takemoto H, Shiozaki H, Yano M, Sekimoto M, Tamura S, Yasuda T, Fujiwara Y, Monden M. Correlation of beta-catenin and cyclin D1 expression in colon cancers. *Oncology* 2001; **61**: 226-233

10 **Brabletz T**, Herrmann K, Jung A, Faller G, Kirchner T. Expression of nuclear beta-catenin and c-myc is correlated with tumor size but not with proliferative activity of colorectal adenomas. *Am J Pathol* 2000; **156**: 865-870

11 **Morrison JA**, Klingelhutz AJ, Raab-Traub N. Epstein-Barr virus latent membrane protein 2A activates beta-catenin signaling in epithelial cells. *J Virol* 2003; **77**: 12276-12284

12 **Sudo M**, Chong JM, Sakuma K, Ushiku T, Uozaki H, Nagai H, Funata N, Matsumoto Y, Fukayama M. Promoter hypermethylation of E-cadherin and its abnormal expression in Epstein-Barr virus-associated gastric carcinoma. *Int J Cancer* 2004; **109**: 194-199

13 **Song BJ**, Park YJ, Kim HS, Kim CN, Chang SH. Expression of beta-catenin and E-cadherin in early gastric cancer. correlation with clinicopathologic parameters. *Korean J Gastroenterol* 2004; **43**: 82-89

14 **Wong SC**, Lo ES, Chan AK, Lee KC, Hsiao WL. Nuclear beta catenin as a potential prognostic and diagnostic marker in patients with colorectal cancer from Hong Kong. *Mol Pathol* 2003; **56**: 347-352

15 **Mingchao** TR, Stockton P, Sun K, Sills RC, Clayton N, Portier M, Flake G. Loss of E-cadherin expression in gastric intestinal metaplasia and later stage p53 altered expression in gastric carcinogenesis. *Exp Toxicol Pathol* 2001; **53**: 237-246

16 **Saeki H**, Tanaka S, Sugimachi K, Kimura Y, Miyazaki M, Ohga T, Sugimachi K. Interrelation between expression of matrix metalloproteinase 7 and beta-catenin in esophageal cancer. *Dig Dis Sci* 2002; **47**: 2738-2742

17 **Utsunomiya T**, Doki Y, Takemoto H, Shiozaki H, Yano M, Inoue M, Yasuda T, Fujiwara Y, Monden M. Clinical significance of disordered beta-catenin expression pattern in human gastric cancers. *Gastric Cancer* 2000; **3**: 193-201

18 **Jones PA**, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999; **21**: 163-167

19 **Baylin SB**, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 2000; **16**: 168-174

20 **Garinis GA**, Menounos PG, Spanakis NE, Papadopoulos K, Karavitis G, Parassi I, Christeli E, Patrinos GP, Manolis EN, Peros G. Hypermethylation-associated transcriptional silencing of E-cadherin in primary sporadic colorectal carcinomas. *J Pathol* 2002; **198**: 442-449

21 **Yoshiura K**, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S. Silencing of the E-cadherin invasion- suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci USA* 1995; **92**: 7416-7419

22 **Carvalho B**, Pinto M, Cirnes L, Oliveira C, Machado JC, Suriano G, Hamelin R, Carneiro F, Seruca R. Concurrent hypermethylation of gene promoters is associated with a MSI-H phenotype and diploidy in gastric carcinomas. *Eur J Cancer* 2003; **39**: 1222-1227

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Effect of Tetrandrine on LPS-induced NF-κB activation in isolated pancreatic acinar cells of rat

Hong Zhang, Yong-Yu Li, Xian-Zhong Wu

Hong Zhang, Department of Pathophysiology, Shaanxi College of Chinese Traditional Medicine, Xianyang 712046, Shaanxi Province, China
Yong-Yu Li, Department of Pathophysiology, Medical College of Tongji University, Shanghai 200092, China
Xian-Zhong Wu, Tianjin Medical University, Tianjin 300070, China
Supported by the National Natural Science Foundation of China, No. 30370643
Correspondence to: Professor Yong-Yu Li, Department of Pathophysiology, Medical College of Tongji University, Shanghai 200092, China. zhangh1227@sohu.com
Telephone: +86-21-65985447
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Abstract

AIM: To investigate the effect of Tetrandrine (Tet) on LPS-induced NF-κB activation and cell injury in pancreatic acinar cells and to explore the mechanism of Tetrandrine preventing LPS-induced acinar cell injury.

METHODS: Male rat pancreatic acinar cells were isolated by collagenase digestion, then exposed to LPS (10 mg/L), Tet (50 μmol/L, 100 μmol/L) or normal media. At different time point (30 min, 1 h, 4 h, 10 h) after treatment with the agents, cell viability was determined by MTT, the product and nuclear translocation of subunit p65 of NF-κB was visualized by immunofluorescence staining and nuclear protein was extracted to perform EMSA which was used to assay the NF-κB binding activity.

RESULTS: LPS induced cell damage directly in a time dependent manner and Tet attenuated LPS-induced cell damage (50 μmol/L, $P < 0.05$; 100 μmol/L, $P < 0.01$). NF-κB p65 immunofluorescence staining in cytoplasm increased and began showing its nuclear translocation within 30 min and the peak was shown at 1 h of LPS 10 mg/L treatment. NF-κB DNA binding activity showed the same alteration pattern as p65 immunofluorescence staining. In Tet group, the immunofluorescence staining in cytoplasm and nuclear translocation of NF-κB were inhibited significantly.

CONCLUSION: NF-κB activation is an important early event that may contribute to inflammatory responses and cell injury in pancreatic acinar cells. Tet possesses the protective effect on LPS-induced acinar cell injury by inhibiting NF-κB activation.

Key words: Tetrandrine; Lipopolysaccharide; Pancreas; Acinar cells; NF-κB

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INTRODUCTION

Many animal and clinical studies have shown that once the progress of acute pancreatitis is initiated, common inflammatory responses are involved. There is a local inflammatory reaction at the site of injury; if marked, this leads to a systematic inflammatory response syndrome (SIRS), and this systemic response is ultimately responsible for the majority of the mortality[1,2]. Lipopolysaccharides (LPS) have been found in the plasma of patients suffering from severe pancreatitis at an early stage of the disease and inflammatory changes resembling acute pancreatitis were described after administration of LPS to several animal species[3,4].

The transcription factor nuclear factor-κB (NF-κB) is a key regulator of cytokine induction. NF-κB represents a family of proteins sharing the Rel homology domain, which bind to DNA as homo- or heterodimers, and activate a multitude of cellular early and stress-related response genes, such as the genes for cytokines, adhesion molecules[5]. NF-κB exists in an inactive form in the cytoplasm of most cells where it binds to an inhibiting protein, IκB. Many stimuli activate NF-κB, including cytokines, LPS and oxidative stress. Stimuli trigger the translocation of NF-κB from cytosol to nucleus where NF-κB binds to its consensus sequence on the promoter-enhancer region of different genes and regulates transcription of specific genes[6]. Recently, some experimental results suggested that the cholecystokinin (CCK) analogue cerulein induced the rapid activation of NF-κB both *in vivo* and in cultured acinar cells *in vitro* and activation of NF-κB preceded pancreatic injury and inflammation[7]. Early blockage of NF-κB activation improved the survival of rats with taurocholate pancreatitis[8].

In our previous study[9,10], we have found that LPS could directly induce the calcium overload, NF-κB activation and cell injury in isolated rat pancreatic acinar cell. Furthermore, NF-κB activation could be attenuated by EGTA, a calcium chelater. Based on these results, we speculate that calcium disorder might take part in the NF-κB activation in isolated rat pancreatic acinar cell and calcium channel blocker (CCB) may be useful for the treatment of acute pancreatitis by influencing NF-κB activation in pancreatic acinar cells.

Tetrandrine (Tet), is a main component of fourstamen stephania root which belongs to traditional Chinese drug and one of natural non-specific CCBs. Tet possesses complicated pharmacological effects and has been used in the therapy of many kinds of disease, such as hypertension, arrhythmia, hepatic fibrosis, *etc*[11]. In former *in vivo* experiments[12,13], we have observed that Tet could improve the pathological alteration of pancreas and lung and decrease the mortality of rats with acute taurocholate pancreatitis. In an attempt to further explore the mechanism of Tet in the treatment of AP, we adopted isolated pancreatic acinar cells and examined whether Tet possesses the protective effect on LPS-induced acinar cell injury by inhibiting NF-κB activation.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats (200-250 g) obtained from Experimental Animal Center of Chinese Academy of Sciences (Grade SPF II Certificate No. SYXK 2002-0023) were fasted for 12 h. *Escherichia coli* LPS (WE coli 055: B5) and MTT were purchased from Sigma Co, USA. Tetrandrine was provided by Pharmacology Department of 2nd Medical University of PLA. NF-κB gel shift assay system kits were provided by Promega Co, USA. [γ-^{32}P] ATP was purchased from Shanghai Isotope Company. Antibody against NF-κB p65 was obtained from Santa Cruz Biotechnology Co, CA. Fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit antibody was purchased from KPL Company, USA. All other chemicals were supplied from local source at the highest purity available. Hepes buffer salt solution (HBSS) (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.13, $NaH_2PO_4 \cdot 2H_2O$ 1.0, *D*-glucose 5.5, HEPES 10, bovine serum albumin 2 g/L, minimum essence medium 2%, *L*-glutamine 2.0, soybean trypsin inhibitor 0.1 g/L, pH adjusted to 7.4 with NaOH 4 mmol/L.

Preparation of isolated pancreatic acinar cells

Pancreatic acinar cells were isolated from male SD rats by collagenase digestion[14]. In brief, after the rat was anesthetized, the pancreas was quickly removed and parenchyma was minced into small fragments and incubated in 10-mL standard buffer containing collagenase V (90 kU/L) at 37℃, and the pancreatic fragments were digested again by collagenase under a shaking condition for 20 min in an incubator. After collagenase digestion, tissue was gently pipetted. Dispersed acini were filtered through a 150-μm nylon mesh, centrifuged 3 times each for 3 min at 100 × g, resuspended with culture media (in HBSS, replacing bovine serum albumin 2 g/L with 10% heat-activated bovine serum) and incubated with 95% O_2, and 5% CO_2.

Cell culture and treatment

Pancreatic acinar cells were planted in 24 and 96 well plates at 37℃ in a CO_2 (50 mL/L) incubator and cultured for 4 h, then exposed to different content of media (10 mg/L LPS, 50 μmol/L Tet and 100 μmol/L Tet or culture media as control) for 30 min, 1 h, 4 h and 10 h respectively.

MTT assay

An MTT assay was employed to assess the viable cell number quantitatively. Briefly, 100 μL of cell suspension (1×10^4 cells) was seeded into 96-well tissue-culture plates. Cells were treated with LPS (10 mg/L), or normal media. Cells in Tet group were treated with 50 μmol/L or 100 μmol/L Tet for 15 min before stimulated by LPS. After treatment with these agents for indicated period, 10 μL MTT (terminal concentration 0.5 g/L) was added into each well, and incubated for 4 h. The formazan crystals were produced by viable cells and dissolved by Me_2SO, and the optical density (OD) of the solution was measured at 490 nm of wavelength. Cell viability was directly proportional to OD value. The viable cell number was expressed as a percentage relative to control cells, measured as $100\% \times OD_{490,\text{ treated}}/OD_{490,\text{ control (at 0 h timepoint)}}$.

NF-κB immunofluorescence studies

The pancreatic acinar cells that were seeded onto 24-well plates and treated as described above respectively for indicated period were used for immunofluorescence staining. First, the glass slips were pretreated with 0.1% poly-lysine and cell suspensions were incubated at room temperature for 60 min. The cells were then fixed in freshly prepared 4% phosphate-buffered paraformaldehyde for 20 min at 4℃. Then they underwent permeabilization with the addition of phosphate-buffered 0.1% Triton X-100 (wt/vol) for 5 min at room temperature and then incubated in PBS containing 1% bovine serum albumin (wt/vol, blocking solution) for 30 min, also at room temperature. Incubation of the cells was done with either rabbit anti-rat NF-κB p65 polyclonal antibodies (1:100 dilution in blocking solution) or with blocking solution alone as negative control for 1 h at room temperature. This was followed by incubation with goat anti-rabbit antibodies (1:100 dilution in blocking solution) conjugated to FITC for 45 min at room temperature. Finally, the slips were mounted and sealed for examination under a confocal microscope.

Electrophoretic mobility shift assay (EMSA) for determination of NF-κB activity

After treatment with stimuli for the above indicated period, pancreatic acinar cells were collected to extract nuclear protein for further examination of the activity of NF-κB by EMSA.

Nuclear protein extraction: Nuclear protein extracts were prepared according to Steinle *et al*[16] with the following modifications. Pancreatic acinar cells (4×10^6) were collected, washed twice with cold phosphate-buffered sa-

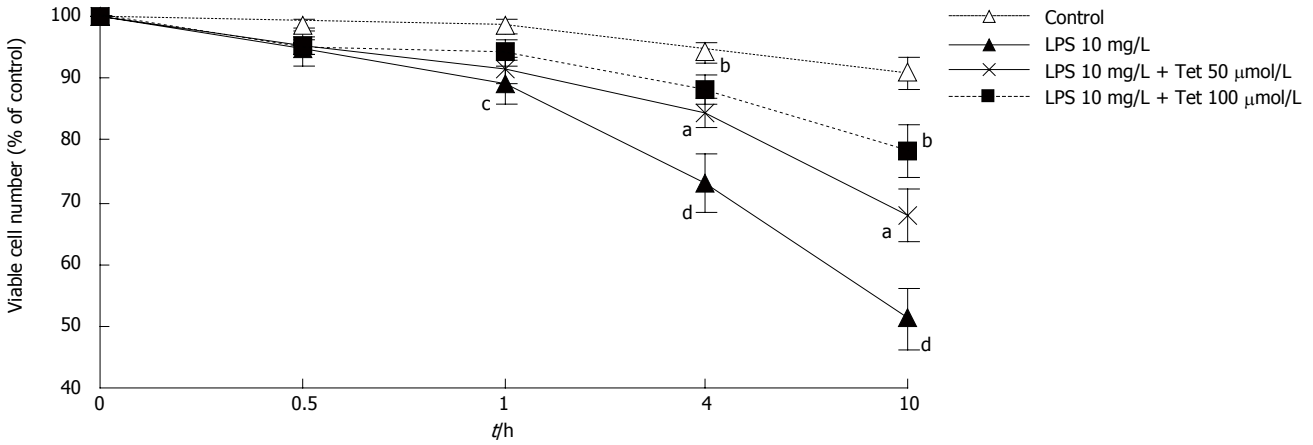

Figure 1 Protective effect of Tet on LPS-induced cell damage in pancreatic acinar cells. Pancreatic acinar cells were treated with LPS 10 mg/L, Tet 50 μmol/L or Tet 100 μmol/L for 1, 4, and 10 h. Results are expressed as percentage of control values (without LPS treatment) at 0 h time-point (n = 3). Mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* LPS group at the same time-point. $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* control group at the same time-point.

line (PBS), homogenized in 1.5% citrate, then centrifuged at 4000 g for 15 min at 4℃, at which point the supernatant was removed. Then 0.3 mL of 0.25 mol/L sucrose-citrate was added into the pellet and mixed to create a nucleic suspension, which was pipetted and paved on the surface of 1.2 mL 0.88 mol/L sucrose-citrate in a tube and centrifuged at 2000 g for 10 min. The resulting pellet was washed twice in 0.05 mol/L Tris-HCl (pH 7.5)-0.15 mol/L NaCl and centrifuged at 2000 g for 10 min at 4℃. After removing the supernatant, an equal volume of KMTD (0.3 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L DTT) was mixed into the pellet and incubated for 1 h. During the incubation, the samples were shaken drastically every 15 min. After incubation, the suspension was centrifuged at 15000 g for 15 min at 4℃. Aliquots of the nuclear protein extracts from supernatant were stored at -70℃. Protein content of the extracts was determined using Lowry method with bovine serum albumin as the standard.

EMSA: Activity of NF-κB was examined by EMSA[15]. The NF-κB oligonucleotide contains DNA binding sites for NF-κB transcription factors. The double-stranded DNA probe sequence is 5'AGT TGA GGG GAC TTT CCC AGG C 3'and antisense 3'TCA ACT CCC CTG AAA GGG TCC G 5' (the binding site is underlined). The 3.5 pmol of the appropriate consensus oligonucleotide was end-labeled with [γ-^{32}P] ATP using T_4 polynucleotide kinase. The ^{32}P-labeled double stranded oligonucleotide was used as a specific probe. For the competition assay, the unrelated oligonucleotide, AP_2 consensus oligonucleotide which lackedκB binding site, was used as a non-specific probe. Nuclear proteins extracted from Hela cell were used as positive control. The nuclear extract equivalent to 5 μg protein was incubated with radiolabeled probe in reaction buffer, and then the mixture was subjected to electrophoresis on 7% acrylamide (wt/vol) gel at 250 V in 0.5 × TBE buffer for 2 h. After being dried, the gel was exposed to X ray film at -70℃ for 48 h.

Statistical analysis

Each n refers to the number of separated experiment. The quantitative data were expressed as mean ± SD and compared using unpaired t-test. The significant differences of incidence of nuclear translocation of NF-κB p65 between LPS group and Tet-pretreated group were analyzed by Chi-square test. $P < 0.05$ was considered significant.

RESULTS

Tet attenuated LPS-induced cell damage

The viable cell number exposed to LPS 10 mg/L decreased with the increase of stimulating time and the difference was significant ($P < 0.05$), compared with control at the same time point. Compared with LPS group, cell mortality in the group pretreated with Tet at 50 μmol/L or 100 μmol/L decreased significantly at each time point (50 μmol/L, $P < 0.05$, 100 μmol/L, $P < 0.01$, Figure 1). It suggests that LPS induced cell damage in a time-dependent manner and Tet could attenuate LPS-induced cell damage.

Tet abrogated LPS-induced NF-κB product and nuclear translocation in pancreatic acinar cells

To monitor its activity at a cellular level, NF-κB p65 antibody was used to recognize the product and nuclear translocation signal of the p65. Active p65 level in cytoplasm and its nuclear translocation in pancreatic acinar cells were monitored by immunofluorescence visualization. The cells untreated with LPS showed a slight exclusive cytoplasmic fluorescence pattern (Figure 2B). In contrast, cytoplasmic fluorescence in pancreatic acinar cells treated with LPS 10 mg/L for 1 h and 4 h increased significantly (Figure 2C and 2D). P65 nuclear translocation was seen in > 60% of pancreatic acinar cells incubated with LPS for 30 min and nuclear staining further increased and reached the peak after 1 h of treatment in the LPS group (nuclear translocation ratio > 90%). In the Tet-pretreated group, the pancreatic acinar cells were pretreated with Tet for 15 min and then stimulated with 10 mg/L LPS for 30 min, 1 h and 4 h. Cytoplasmic fluorescence in pancreatic acinar cells was impaired (Figure 2E and 2F) and P65 nuclear translocation was little seen, compared with that in the LPS group ($P < 0.05$ or $P < 0.01$, Table 1).

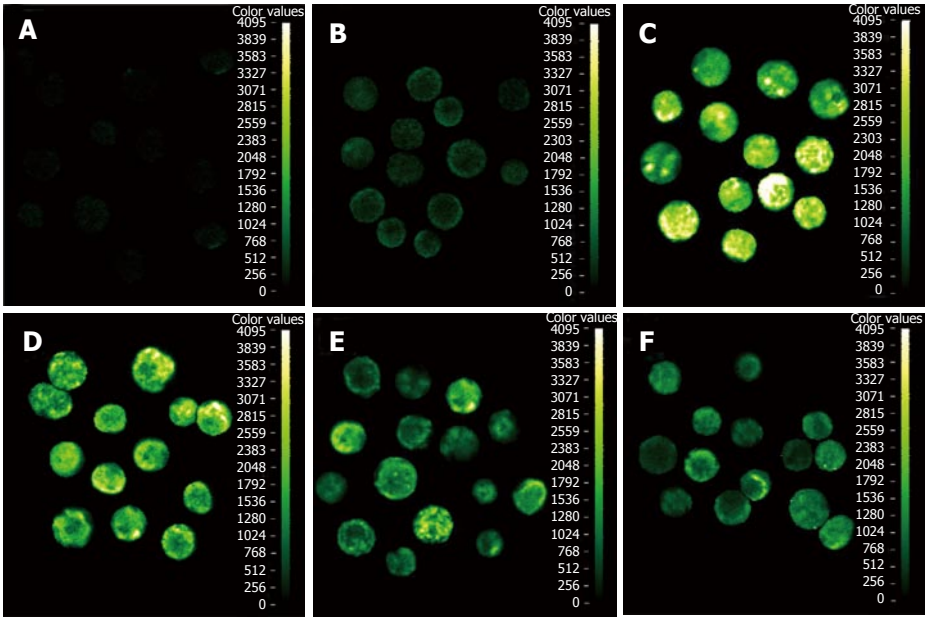

Figure 2 Tet prevented LPS-induced nuclear translocation of p65 in isolated pancreatic acinar cells. P65 was visualized by indirect immunofluorescence staining using rabbit anti-p65 polyclonal antibodies (1:100) which only recognized NF-κB p65. Goat anti-rabbit antibodies (1:100) conjugated to FITC was performed, and visualized under a confocal microscope. Nuclear p65 was observed at 1 h (**C**), 4 h (**D**) after treatment with LPS (10 mg/L) but not in saline-treated controls at 1 h (**B**), nor in negative control (**A**). Nuclear localization of p65 and the fluorescence in cytoplasm were markedly reduced by Tet 100 μmol/L at 1 (**E**), 4 h (**F**), compared with that of LPS group at the same time (Original magnification × 400).

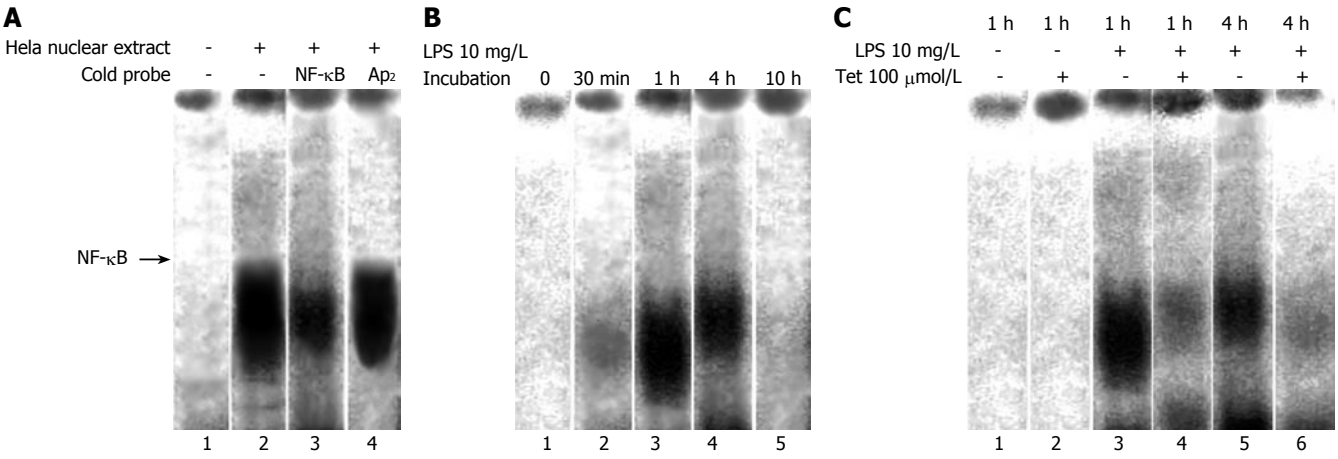

Figure 3 **A**: Effect of LPS on NF-κB activity in pancreatic acinar cell. Lane 1: control; Lane 2: treated with LPS 10 mg/L for 1 h; Lane 3: treated with LPS 10 mg/L for 1 h + cold NF-κB probe; Lane 4: treated with LPS 10 mg/L for 1 h + labeled AP_2 probe; **B**: The time course of LPS-induced NF-κB activity in pancreatic acinar cell. NF-κB activity in pancreatic acinar cell stimulated by LPS 10 mg/L for indicated periods of time. Lane 1: 0 min; Lane 2: 30 min; Lane 3: 1 h; Lane 4: 4 h; Lane 5: 10 h; **C**: Effect of Tet on LPS-induced NF-κB activity in pancreatic acinar cell. Lane 1: control; Lane 2: treated with Tet 100 μmol/L for 1 h; Lane 3: treated with LPS 10 mg/L for 1 h; Lane 4: treated with LPS 10 mg/L + Tet 100 μmol/L for 1 h; Lane 5: treated with LPS 10 mg/L for 4 h; Lane 6: treated with LPS 10 mg/L + Tet 100 μmol/L for 4 h.

Tet interfered with LPS-induced NF-κB activation: EMSA was performed to detect the binding activity of NF-κB in different group. Pancreatic acinar cells were incubated with LPS or Tet and nuclear extracts were prepared and incubated with a ^{32}P-labeled DNA oligonucleotide containing the recognition site of NF-κB. The specificity of the shift bands was verified by competition assays: The shift bands were suppressed by the incubation with cold NF-κB probe and not influenced by non-specific competition with labeled AP_2 probe (Figure 3A). Very little NF-κB binding activity was detected under the unstimulated condition and LPS induced NF-κB binding activity increased in a time dependent manner and NF-κB binding activity was maximal at 1 h after incubation of LPS in 10 mg/L (Figure 3B).

The effect of Tet on LPS-induced NF-κB activation was assessed in pancreatic acinar cells. It was found that in the presence of Tet 100 μmol/L, the optical density and volume of NF-κB shift band markedly decreased than that of LPS group at the same time (Figure 3C).

Table 1 Effect of Tet on LPS-induced nuclear translocation of NF-κB p65 in pancreatic acinar cells

Group	Ratio of nuclear translocation (%)		
	30 min	1 h	4 h
LPS 10 mg/L	60.9	90.5	44.7
Tet 50 μmol/L + LPS	34.7[a]	34.5[b]	18.9[b]
Tet 100 μmol/L+ LPS	28.6[b]	32.1[b]	17.3[b]

Pancreatic acinar cells were pretreated with 50 μmol/L or 100 μmol/L Tet for 15 min and then stimulated with 10 mg/L LPS for 30 min, 1 h and 4 h. In the Tet-pretreated group, cytoplasmic fluorescence in pancreatic acinar cells was impaired (Figure 2E and 2F) and P65 nuclear translocation was little seen, compared with that in the LPS group. [a]$P < 0.05$, [b]$P < 0.01$ *vs* LPS group at the same time-point.

DISCUSSION

Lipopolysaccharide (LPS, endotoxin) of the gram negative bacteria outer wall plays a central role in the pathophysiology of the sepsis syndrome[3]. Many experiments demonstrated it is related to the pathogenesis of acute pancreatitis[4,15,16].

LPS induces NF-κB activation in many cells, such as canine tracheal smooth muscle cells, primary rat microglia or monocyte of humans[17-19]. In our previous study[9], we have found that LPS induced the increase of NF-κB binding activity by EMSA and p65 subunit of NF-κB translocation to nuclei by immunofluoresence. However, it is unknown whether there is a more primary factor which precedes the LPS-induced NF-κB activation. Our other study[10] revealed that the first alteration measured after exposing pancreatic acinar cells to LPS system was an increase in the $[Ca^{2+}]_i$ which appeared within several hundreds of second. The increase in $[Ca^{2+}]_i$ preceded all the other pathological events in the progress of LPS-induced pancreatic acinar cells damage. Intracellular calcium overload exerted an important effect on LPS-induced pancreatic acinar cells damage and egtazic acid, a Ca^{2+} chelate could attenuate the damage by inhibiting Ca^{2+} influx. The result suggested that the disorder of calcium homeostasis in pancreatic acinar cells was an important mediator of LPS-induced cell damage. Tando *et al* observed that caerulein-induced NF-κB/Rel activation requires both Ca^{2+} and protein kinase C as messenger[5]. We adopted calcium channel blocker (CCB) to observe its effects on the NF-κB activation and viability of isolated rat pancreatic acinar cell.

In our present study, we found that LPS was able to induce the damage in intact pancreatic acinar cells directly at 1 h and the mortality of cell increased with the increase of concentration and stimulating time of LPS. The increase of NF-κB activity preceded the pathological alteration in the progress of LPS-induced pancreatic acinar cells damage. Moreover, a typical CCB, Tet could abrogate LPS-induced NF-κB nuclear translocation, interfere with the DNA binding activity of NF-κB and attenuate cell damage of pancreatic acinar cells. The results show that NF-κB activation is an important early event that may contribute to inflammatory response and cell injury in pancreatic acinar cells and Tet possesses the protective effect on LPS-induced acinar cell injury by inhibiting NF-κB activation. These findings will be helpful to explain the mechanism of Tet in the treatment of AP at the biological level.

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REFERENCES

1 **Bhatia M**, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. Inflammatory mediators in acute pancreatitis. *J Pathol* 2000; **190**: 117-125
2 **Gloor B**, Uhl W, Tcholakov O, Roggo A, Muller CA, Worni M, Büchler MW. Hydrocortisone treatment of early SIRS in acute experimental pancreatitis. *Dig Dis Sci* 2001; **46**: 2154-2161
3 **Raetz CR**. Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. *J Bacteriol* 1993; **175**: 5745-5753
4 **Exley AR**, Leese T, Holliday MP, Swann RA, Cohen J. Endotoxaemia and serum tumour necrosis factor as prognostic markers in severe acute pancreatitis. *Gut* 1992; **33**: 1126-1128
5 **Tando Y**, Algül H, Wagner M, Weidenbach H, Adler G, Schmid RM. Caerulein-induced NF-kappaB/Rel activation requires both Ca2+ and protein kinase C as messengers. *Am J Physiol* 1999; **277**: G678-G686
6 **Steinle AU**, Weidenbach H, Wagner M, Adler G, Schmid RM. NF-kappaB/Rel activation in cerulein pancreatitis. *Gastroenterology* 1999; **116**: 420-430
7 **Grisham MB**. NF-kappaB activation in acute pancreatitis: protective, detrimental, or inconsequential? *Gastroenterology* 1999; **116**: 489-492
8 **Satoh A**, Shimosegawa T, Fujita M, Kimura K, Masamune A, Koizumi M, Toyota T. Inhibition of nuclear factor-kappaB activation improves the survival of rats with taurocholate pancreatitis. *Gut* 1999; **44**: 253-258
9 **Zhang H**, Li Y, Wang S, Zhang K, Li L, Wu X. LPS-induced NF-kappa B activation requires Ca2+ as a mediator in isolated pancreatic acinar cells of rat. *Chin Med J (Engl)* 2003; **116**: 1662-1667
10 **Zhang H**, Li YY, Wang SN, Zhang KH, Wu XZ. Effects of lipopolysaccharides on calcium homeostasis in isolated pancreatic acinar cells of rat. *Acta Pharmacol Sin* 2003; **24**: 790-795
11 **Jiang JM**, Dai DZ. Research progression of tetrandrine antagonize calcium channel. *Zhongguo Yaolixue Tongbao* 1998; **14**: 297-300
12 **Zhang H**, Li YY, Bai JL, Yang YH. Study on therapeutic effect of tetrandrine on acute pancreatitis in rats and its mechanism. *Zhongguo Zhongxiyi Jiehe Zazhi* 2002; **22**: 125-127
13 **Zhang H**, Li YY. Therapeutic effect of tetrandrine on injury of pancreas and lung of rats with experimental acute pancreatitis. *Tongji Daxue Xuebao* (Yi Xue Ban) 2002; **22**: 363-367
14 **Kitagawa M**, Williams JA, De Lisle RC. Amylase release from streptolysin O-permeabilized pancreatic acini. *Am J Physiol* 1990; **259**: G157-G164
15 **Siebenlist U**, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* 1994; **10**: 405-455
16 **Laine VJ**, Nyman KM, Peuravuori HJ, Henriksen K, Parvinen M, Nevalainen TJ. Lipopolysaccharide induced apoptosis of rat pancreatic acinar cells. *Gut* 1996; **38**: 747-752
17 **Yang CM**. Lipopolysacchride induction of cyclooxygenase-2 expression is mediated via mitogen-activated protein kinase and nuclear factor-κB pathways in canine tracheal smooth muscle cells. *FASEB J* 2002; **16**: A1147-1152
18 **Holmes MM**, Southerland J, Downey C,Baldwin AS, Offenbacher S. Synnergy of AGEs and P.g. LPS-induced NF-κB activation and Cox-2 expression. *FASEB J* 2002; **16**: A233-239
19 **Hill DB**, Barve S, Joshi-Barve S, McClain C. Increased monocyte nuclear factor-kappaB activation and tumor necrosis factor production in alcoholic hepatitis. *J Lab Clin Med* 2000; **135**: 387-395

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
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RAPID COMMUNICATION

Investigation on correlation between expression of CD58 molecule and severity of hepatitis B

Li Sheng, Jie Li, Bao-Tai Qi, Yu-Qiang Ji, Zhao-Jun Meng, Ming Xie

Li Sheng, Jie Li, Bao-Tai Qi, Yu-Qiang Ji, Zhao-Jun Meng, Ming Xie, Department of Pathogenic Microbiology and Immunology, Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Supported by a grant from the National Natural Science Foundation of China, No. 30371321
Correspondence to: Ming Xie, Associate Professor, Department of Pathogenic Microbiology and Immunology, Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. xieming@mail.xjtu.edu.cn
Telephone: +86-29-82655182
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Abstract

AIM: To investigate the correlation between expression of CD58 and severity of hepatitis B.

METHODS: The level of soluble CD58 (sCD58) in serum of patients with hepatitis B was detected by enzyme-linked immunosorbent assay. The level of expression of membrane CD58 molecule in PBMC was detected by direct immunofluorescence. The levels of serumal TBIL, DBIL, IBIL, ALT and AST were detected by the automated biochemistry analyzer as well.

RESULTS: The levels of sCD58 in serum and membrane CD58 molecule in PBMC of patients with hepatitis B were significantly higher than that in normal controls ($P < 0.05$). Level of CD58 was related to the levels of serumal TBIL, DBIL, IBIL, ALT and AST.

CONCLUSION: The level of CD58 molecule (in both serum and PBMC form) of patients with hepatitis B is related to the degree of liver damage.

Key words: Hepatitis B; CD58; Liver damage

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INTRODUCTION

Hepatitis B is caused by Hepatitis B virus (HBV), but the pathogenesis of hepatitis B is not well understood. HBV infects human liver cells but has no direct cytopathic effect on these cells. Therefore, it is unlikely that direct viral cytotoxicity is the primary cause of pathology in vivo. Several studies have suggested that hepatitis B may be mediated in part by immunopathologic mechanisms. As one of the intercellular adhesion molecules, CD58 provided co-stimulatory signals for the activation of T lymphocyte, it plays an important role in promoting the adhesion of T cells to targeted cells[1-3]. In this study, we used double antibody sandwich ELISA and direct immunofluorescece to analyze the levels of sCD58 in serum and the expression of CD58 on the surface of PBMC of patients with hepatitis B, and compared with those levels of healthy controls to evaluate the role of CD58 in the pathogenesis of hepatitis B.

MATERIALS AND METHODS

Sample collection and processing

Forty-three patients with hepatitis B are selected from outpatients and inpatients of the Department of Infectious Diseases of First Hospital of Xi'an Jiaotong University and Second Hospital of Xi'an Jiaotong University. The patients were divided into four groups, namely mild chronic hepatitis B group ($n = 12$), moderate chronic hepatitis B group ($n = 11$), severe chronic hepatitis B group ($n = 10$) and severe hepatitis B group ($n = 10$). Eleven healthy persons were taken as normal control group. The diagnostic code for Hepatitis B which edited by 5th Chinese Academic committee of Infection Disease and Parasite in 2000 was used as the classification criteria.

Detection of the sCD58 in serum

The serum samples were diluted 1:50 with assay buffer (5 μL sample + 245 μL assay buffer), and then the levels of sCD58 in the diluted samples were detected by use of double antibody sandwich ELISA following the kit instructions (Australia Science Laboratory company). The standard wells were designated by the instruction as well. The OD value of each well of the samples and the sCD58 standards was read out on an EL311 autoplate reader (BIO-TEK Instruments, American) with a test wavelength of 450 nm and a reference wavelength of 620 nm; hence the optimal concentration of sCD58 could be determined. Blank wells were used as background control.

Detection of membrane CD58 in PBMC

One ml of PBMC sample was mixed with 10 μL of

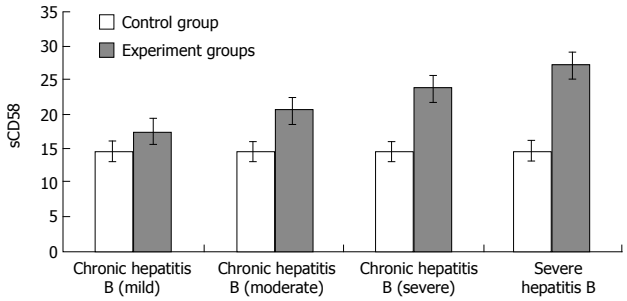

Figure 1 Content of CD58 in serum.

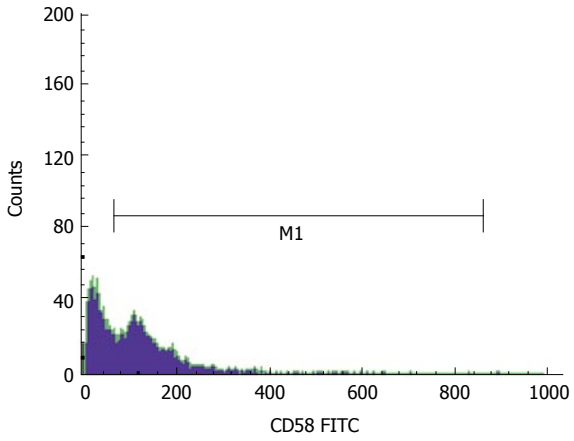

Figure 2 Content of membrane CD58 molecule in mild chronic hepatitis B.

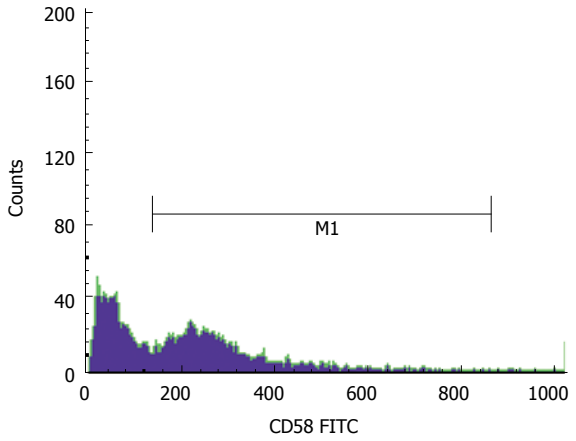

Figure 3 Content of membrane CD58 molecule in moderate chronic hepatitis B.

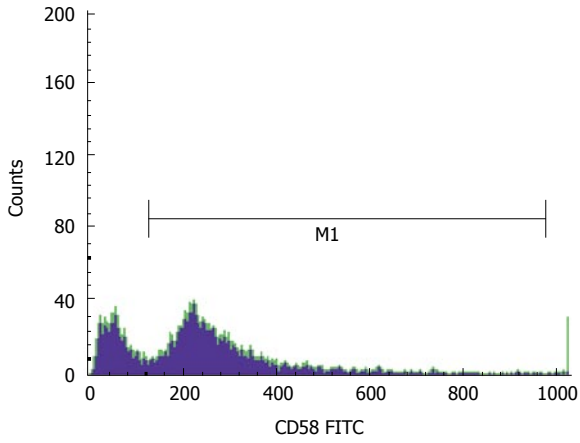

Figure 4 Content of membrane CD58 molecule in severe chronic hepatitis B.

mouse anti-human CD58 antibody marked by fluorescence FITC (American Southern Biotech), incubated in the dark at room temperature, washed twice with PBS, and centrifuged at 2500 r/min before abandoning supernatant. One ml of PBS-formaldehyde was added to fix cells, and CD58 positive cells were dectected by using FACS Calibur flow cytometer.

Detection of the liver function

One mL serum sample was used to test the levels of TBIL, DBIL, IBIL, ALT and AST by the CHEMIX-180 automated biochemistry analyzer (Japanese SYSMEX Corporation).

Statistical Analysis

The significance among different groups was examined by one-way analysis of variance followed by tow-sample Student's *t*-test. Differences between groups were considered significant if probability value of $P < 0.05$ were obtained. The data were presented by means ± SD.

RESULTS

Content of sCD58 in serum

The levels of sCD58 of patient with HBV infection were significantly higher than that of normal control ($P < 0.05$); and the difference among the groups were significant ($P < 0.05$) (Figure 1).

Content of membrane CD58 molecule

The results showed that the level of membrane CD58 molecule in PBMC of patients with HBV infection was significantly higher than that of the normal group and the differences among the groups were significant ($P < 0.05$). The levels of membrane CD58 molecule increased significantly in an order from light chronic hepatitis B, medium group, severe group and severe hepatitis B. The membrane CD58 molecule in PBMC might relate to the severity of the disease (Figures 2-5, Table 1).

DISCUSSION

CD58 is also called lymphocyte function associated antigen-3 (LFA-3)[4-6], which belongs to the CD2 family. As an important co-stimulating molecule, CD58 plays an important role in promoting the adhesion of T cells to targeted cells, and enhancing the recognition and sensitivity of T lymphocyte to the superantigen[7-9]. CD58 promotes hyperplasia and activation of T cell[1,2], promotes T cells soak inflammatory parts and takes part in signal transmission of T cells. Combined with CD2 molecules on the surface of NK cells, CD58 increases the adhesion between NK cells and target cells, activates NK cells[10,11], and increases the toxin of the cells[12,13]. After integrating

Table1 The liver function

Group	*n*	TBIL (μmol/L)	DBIL (μmol/L)	IBIL (μmol/L)	ALT (IU/L)	AST (IU/L)
Normal control	11	11.25 ± 2.14	3.12 ± 1.54	6.41 ± 1.85	25.19 ± 2.58	19.57 ± 3.06
Chronic hepatitis B (mild)	12	15.14 ± 3.26[a]	15.92 ± 2.05[a]	10.39 ± 2.63[a]	63.33 ± 3.68[a]	46.67 ± 9.81[a]
Chronic hepatitis B (moderate)	11	39.21 ± 8.73[a]	25.21 ± 7.11[a]	23.74 ± 3.87[a]	98.21 ± 18.90[a]	114.43 ± 12.80[a]
Chronic hepatitis B (severe)	10	105.33 ± 17.67[a]	49.88 ± 8.62[a]	50.86 ± 16.05[a]	221.61 ± 18.19[a]	157.01 ± 22.54[a]
Severe hepatitis B	10	143.57 ± 23.15[a]	75.26 ± 6.56[a]	117.35 ± 15.27[a]	116.73 ± 28.57	94.82 ± 41.49

[a]$P < 0.05$, compared with control group.

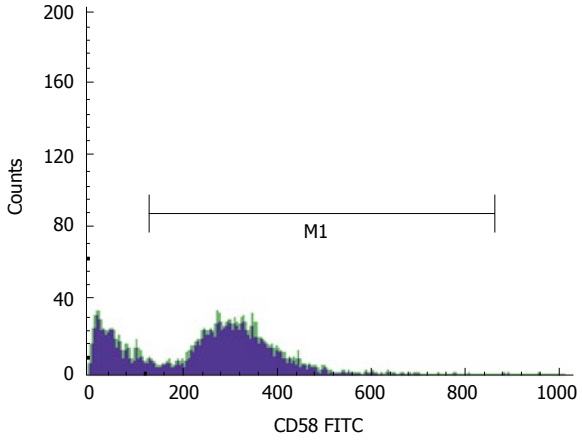

Figure 5 Content of membrane CD58 molecule in severe hepatitis B.

with activated T cells, CD58 and CD2 facilitate interferon γ and IL-2 mRNA record and translate, differentiate $CD4^+$T to Th1 and further initiate the immune response of the cell[3,14]. Some researchers proved that integrated with matching cells, CD58 may boost the ability of activated T cells and NK cells[15,16].

Our experiment showed that the levels of sCD58 in serum and membrane CD58 molecule in PBMC of patients with HBV infection were significantly higher than that of the normal group. The levels of CD58 varied from different groups of patients with hepatitis B, correlated to the severity of the disease. The results also showed that the percentage of $CD58^+$ cell of patients with hepatitis B might be related with TBIL, DBIL, IBIL, ALT and AST, which prove the expression of CD58 is closely correlated with the liver damage. According to the results of the research, the increased levels of sCD58 molecule in serum and membrane CD58 molecule in PBMC of patients with hepatitis B could enhance the adhesion of APC and T cells to identify antigen[17,18]. This study proved that the combination of CD58 and CD2 activated T cells was similar to the way of TCR/CD3. This might lead to a series of responses in the cells, including the increase of the concentration of $Ca2^+$, the activation of PKC, several limpid genes in the resting cells such as IL-2 and IFN-γ being recorded and the transformation of T cells from G0 stage to S stage. The T cells activated by integration of CD58 and CD2 would increase the record and translate of IL-2 and IFN-γ mRNA, and then differentiate into Th1 which would enhance the cell immune response. Combined with CD2 molecules on the surface of NK cells, CD58 could activate NK cells and increase the cytotoxic effect[19]. And this would contribute to the organisms to eliminate HBV. The cytotoxic effect of the immune cells might be implemented by releasing PF or inducing apoptosis of CD95 cells[20].

In summary, this study showed that the sCD58 in serum and membrane CD58 molecule in PBMC of the patients with hepatitis B is related to the severity of the disease and the liver damage. CD58 might enhance the elimination of viruses through activating T and NK cells and promoting cell immune response. However, this would also lead to the damage of liver cells.

REFERENCES

1 **Oh S**, Hodge JW, Ahlers JD, Burke DS, Schlom J, Berzofsky JA. Selective induction of high avidity CTL by altering the balance of signals from APC. *J Immunol* 2003; **170**: 2523-2530
2 **Lopez RD**, Waller EK, Lu PH, Negrin RS. CD58/LFA-3 and IL-12 provided by activated monocytes are critical in the in vitro expansion of CD56+ T cells. *Cancer Immunol Immunother* 2001; **49**: 629-640
3 **Le Guiner S**, Le Dréan E, Labarrière N, Fonteneau JF, Viret C, Diez E, Jotereau F. LFA-3 co-stimulates cytokine secretion by cytotoxic T lymphocytes by providing a TCR-independent activation signal. *Eur J Immunol* 1998; **28**: 1322-1331
4 **Henniker AJ**. CD58 (LFA-3). *J Biol Regul Homeost Agents* 2001; **15**: 190-192
5 **Elangbam CS**, Qualls CW Jr, Dahlgren RR. Cell adhesion molecules--update. *Vet Pathol* 1997; **34**: 61-73
6 **Hahn WC**, Menu E, Bothwell AL, Sims PJ, Bierer BE. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. *Science* 1992; **256**: 1805-1807
7 **Geppert TD**, Lipsky PE. Immobilized anti-CD3-induced T cell growth: comparison of the frequency of responding cells within various T cell subsets. *Cell Immunol* 1991; **133**: 206-218
8 **Shaw S**, Shimizu Y. Two molecular pathways of human T cell adhesion: establishment of receptor-ligand relationship. *Curr Opin Immunol* 1988; **1**: 92-97
9 **Halvorsen R**, Leivestad T, Gaudernack G, Thorsby E. Accessory cell-dependent T-cell activation via Ti-CD3. Involvement of CD2-LFA-3 interactions. *Scand J Immunol* 1988; **28**: 277-284
10 **Barber DF**, Long EO. Coexpression of CD58 or CD48 with intercellular adhesion molecule 1 on target cells enhances adhesion of resting NK cells. *J Immunol* 2003; **170**: 294-299
11 **Fletcher JM**, Prentice HG, Grundy JE. Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV-induced down-regulation of cell surface class I HLA. *J Immunol* 1998; **161**: 2365-2374
12 **Grosenbach DW**, Schlom J, Gritz L, Gómez Yafal A, Hodge

JW. A recombinant vector expressing transgenes for four T-cell costimulatory molecules (OX40L, B7-1, ICAM-1, LFA-3) induces sustained CD4+ and CD8+ T-cell activation, protection from apoptosis, and enhanced cytokine production. *Cell Immunol* 2003; **222**: 45-57

13 **Gollob JA**, Ritz J. CD2-CD58 interaction and the control of T-cell interleukin-12 responsiveness. Adhesion molecules link innate and acquired immunity. *Ann N Y Acad Sci* 1996; **795**: 71-81

14 **Gollob JA**, Li J, Kawasaki H, Daley JF, Groves C, Reinherz EL, Ritz J. Molecular interaction between CD58 and CD2 counterreceptors mediates the ability of monocytes to augment T cell activation by IL-12. *J Immunol* 1996; **157**: 1886-1893

15 **Gollob JA**, Li J, Reinherz EL, Ritz J. CD2 regulates responsiveness of activated T cells to interleukin 12. *J Exp Med* 1995; **182**: 721-731

16 **Chen CM**, Li SC, Lin YL, Hsu CY, Shieh MJ, Liu JF. Consumption of purple sweet potato leaves modulates human immune response: T-lymphocyte functions, lytic activity of natural killer cell and antibody production. *World J Gastroenterol* 2005; **11**: 5777-5781

17 **Polese L**, Angriman I, Giuseppe DF, Cecchetto A, Sturniolo GC, Renata D, Scarpa M, Ruffolo C, Norberto L, Frego M, D' Amico DF. Persistence of high CD40 and CD40L expression after restorative proctocolectomy for ulcerative colitis. *World J Gastroenterol* 2005; **11**: 5303-5308

18 **Qiu WH**, Zhou BS, Chu PG, Chen WG, Chung C, Shih J, Hwu P, Yeh C, Lopez R, Yen Y. Over-expression of fibroblast growth factor receptor 3 in human hepatocellular carcinoma. *World J Gastroenterol* 2005; **11**: 5266-5272

19 **Han YN**, Yang JL, Zheng SG, Tang Q, Zhu W. Relationship of human leukocyte antigen class II genes with the susceptibility to hepatitis B virus infection and the response to interferon in HBV-infected patients. *World J Gastroenterol* 2005; **11**: 5721-5724

20 **Thompson CB**. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**: 1456-1462

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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RAPID COMMUNICATION

Expression of angiopoietins, Tie2 and vascular endothelial growth factor in angiogenesis and progression of hepatocellular carcinoma

Zhong-Lin Zhang, Zhi-Su Liu, Quan Sun

Zhong-Lin Zhang, Zhi-Su Liu, Quan Sun, Department of General Surgery, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China
Correspondence to: Professor Zhi-Su Liu, Department of General Surgery, Zhongnan Hospital, Wuhan University, No. 169, Donghu Road, Wuhan 430071, Hubei Province, China. zhangzhonglin221@sina.com
Telephone: +86-27-67813007 Fax: +86-27-87330795
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Abstract

AIM: To investigate the significance of angiopoietins, Tie2 and vascular endothelial growth factor (VEGF) expression in the angiogenesis and progress of hepatocellular carcinoma (HCC).

METHODS: Fresh surgically resected specimens of HCC and noncancerous liver (NCL) tissue from 38 patients with HCC were obtained, and expression of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Tie2, and VEGF messenger RNA (mRNA) was examined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Expression pattern of each gene in HCC and NCL tissue specimens was compared and the potential role and interaction in angiogenesis of HCC were analyzed. Genes' expression level and its relationship with tumor's clinicopathological parameters were also investigated. Immunohistochemical staining of CD34 was performed to determine the microvessel density (MVD) and Ang-2/Ang-1 ratio was calculated. Relationships between Ang-2/Ang-1 ratio, VEGF and MVD and clinicopathological features were also tested so as to evaluate their significance in the progression of HCC.

RESULTS: Ang-2 and VEGF mRNAs in HCC were significantly higher than those in NCL tissue ($P < 0.05$), whereas the Ang-1 and Tie2 mRNAs showed no statistical significance ($P > 0.05$), though slightly lower level of Ang-1 mRNA in HCC was observed. Ang-2/Ang-1 ratio and VEGF were both positively correlated to MVD. The Ang-2/ Ang-1 ratio, Ang-2 and VEGF were all associated with tumor's clinicopathological parameters ($P < 0.05$) except for histological grades ($P > 0.05$). Ang-1 and Tie2 levels in different clinicopathological groups were not significantly different ($P > 0.05$).

CONCLUSION: Dominant Ang-2 expression against Ang-1 through Tie2 receptor in the presence of VEGF plays a critical role in initiating early neovascularization and transformation of noncancerous liver to hepatocellular carcinoma. Its consequently constant operation in formed HCC induces further angiogenesis and progression of HCC.

Key words: Hepatocellular carcinoma; Vascular endothelial growth factor; Angiopoietin; Tie2; Angiogenesis; Neovascularization

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INTRUDUCTION

Hepatocellular carcinoma (HCC) is one of the most malignant tumors in the tropics and the Far East, including China. It is the fourth most common cause of cancer death and accounts for 53% of all liver cancer deaths worldwide[1]. HCC is also a hypervascular carcinoma. It is believed that angiogenesis contributes to its malignant biological characteristics such as invasion and high rates of recurrence and metastasis[2-4]. Angiogenesis is a neovascularization process during which endothelial cells of the pre-existing capillaries proliferate and migrate to form new vascular tips or so-called "vascular sprouts" or "endothelial buds"[5]. The growth, invasion and metastasis of malignant tumors depend on the process of angiogenesis. There is evidence that solid tumors do not grow beyond 2-3 mm^3 in volume when vascular sprouts are blocked[6].

Angiogenesis is a very complicated network which is closely regulated by many angiogenic factors. Vascular endothelial growth factor (VEGF) and angiopoietin are the two most important regulators. The latter is a novel family of angiogenic factors including Ang-1, Ang-2, Ang-3, and Ang-4, which have been isolated and identified as a group of ligands of the tyrosine kinase Tie2 receptor[7,8]. Ang-1 and Ang-2 have been reported as the most potent regulators for neovascularization[9] and are the activator and an-

tagonist of Tie2 receptor, where binding of Ang-1 causes autophosphorylation of Tie2 whereas Ang-2 binding suppresses the autophosphorylation[9,10]. It was reported that proper regulation of tyrosine kinase Tie2 is absolutely required for normal vascular development, apparently by regulating vascular remodeling and maturation[11]. Ang-1 helps to maintain and stabilize maturation vessels by promoting interaction between endothelial cells and support cells, such as pericytes. Knockout mice deficient in Ang-1 develop severe vascular defect and die in uterus, similar to Tie2 deficient mice[12]. Ang-2 acts as an alternative ligand for Tie2 and binds to Tie2 with similar affinity, but competitively antagonizes Ang-1 effects with blockage of Tie2 phosphorylation and activation. Functionally, transgenic mice over-expressing Ang-2 show even more severe vascular defects as the Ang-1 or Tie2 deficient mice[13]. In the presence of VEGF, vessel destabilization caused by Ang-2 has been hypothesized to induce angiogenic response, whereas in the absence of VEGF, Ang-2 leads to vessel regression[13,14].

Although the exact role of angiopoietin/Tie2 system remains enigmatic, there is evidence that this system in the presence of VEGF is important for the initiation of angiogenesis and vascular sprouting in tumors[15]. It was recently reported that VEGF and aniopoietin/Tie2 system play a key role in the transformation of normal lung to non-small cell lung carcinoma[16]. However, their exact role in the initiation and development of HCC is still unclear. In this study, we investigated the expression of Ang-1, Ang-2, Tie2 and VEGF by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Thirty-eight specimens of HCC and noncancerous liver (NCL) tissues were examined in an attempt to definite their role.

MATERIALS AND METHODS

Patients and specimens

Fresh surgically resected specimens of HCC and noncancerous liver tissue were obtained from 38 patients with HCC who underwent partial hepatectomy in the Department of General Sugery of Zhongnan Hospital, Wuhan University (Wuhan, China) from 2003 to 2005. Demographic data of all patients are shown in Table 1. Thirty-four patients suffered from hepatitis and liver cirrhosis at different extent. Diagnosis of all patients was confirmed by histological examination after operation, and the noncancerous liver tissue was resected. Fully informed consent was obtained from all patients, and the study was performed in accordance with the guidelines of the Helsinki Declaration of 1975 amended in 1983. Those who accepted any therapy or accompanied any other severe complications and those with metastatic liver tumor were excluded. Some samples were frozen in liquid nitrogen immediately after resection and the remaining were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin for pathological examination. Histological grade was classified according to the criteria of Edmondson and Steiner.

Real-time semiquantitative RT-PCT assay

Total RNA was extracted with Trizol reagent (Promega, USA) following the manufacturer's instructions and quantitated by absorbance analysis at 260 nm. First-strand cDNA was synthesized using first standard buffer, dNTP mixture containing each deoxynucleotide triphosphate base dithiothreitol and moloney murine leukemia virus RT (GIBCO, BRIL) as previously described[17]. Four-fold dilution of the products was used for PCR in a Rotor-Gene2000 real-time PCR machine (Corbett Research, Australia). Primers of each gene are shown in Table 2 as previously described[10]. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal standard. Reverse transcription was performed at 50℃ for 30 min. The samples were subjected to PCR analysis using the following cycling parameters: at 95℃ for 10 min, then at 95℃ for 15 s and at 60℃ for 1 min for 40 cycles. Negative controls (cDNA-free solutions) were included in each reaction. Standard reaction curve was analyzed by Rotor-Gene 5.0 (Corbett Research)

Table 1 Demographic data and clinicopathological features in 38 cases of HCC

No.	Sex	Age	Tumor size (cm)	Tumor capsule	Metastasis	Portal vein invasion	Histological grade	Liver cirrhosis
1	M	51	< 5	+	-	-	G2	-
2	M	49	< 5	+	-	-	G3	+
3	M	31	> 10	-	-	-	G2	+
4	M	59	5-10	+	-	-	G3	+
5	F	55	> 10	-	-	-	G1	+
6	M	65	> 10	-	-	+	G3	+
7	M	56	> 10	-	+	+	G2	+
8	M	51	> 10	-	-	+	G3	+
9	F	49	< 5	+	-	-	G1	-
10	M	50	5-10	-	-	-	G2	+
11	M	53	> 10	-	-	+	G2	+
12	M	43	< 5	+	-	-	G3	+
13	M	36	5-10	+	-	-	G2	+
14	F	46	5-10	-	-	-	G1	+
15	F	45	5-10	-	-	-	G1	+
16	M	52	5-10	-	-	-	G2	+
17	F	50	5-10	-	-	-	G1	+
18	F	52	< 5	+	-	-	G1	+
19	F	61	> 10	-	+	+	G1	+
20	M	36	5-10	-	-	-	G2	+
21	M	46	< 5	+	-	-	G2	+
22	M	48	> 10	-	-	+	G2	+
23	F	63	< 5	+	-	-	G1	+
24	M	35	5-10	+	-	-	G2	-
25	M	74	> 10	-	+	+	G3	+
26	F	55	5-10	-	-	-	G1	+
27	M	59	5-10	-	-	-	G2	+
28	M	30	< 5	+	-	-	G3	+
29	F	56	5-10	-	-	-	G1	+
30	M	60	5-10	-	-	-	G2	+
31	F	65	< 5	+	-	-	G1	+
32	M	59	> 10	-	+	+	G2	+
33	M	61	> 10	-	+	+	G2	+
34	M	55	< 5	-	-	-	G2	-
35	M	48	5-10	+	-	-	G3	+
36	M	49	> 10	-	-	+	G3	+
37	F	50	5-10	-	-	-	G1	+
38	M	58	> 10	-	+	+	G3	+

Table 2 Primer sequences of each gene for real-time RT-PCR assay

Target gene	Sense	Antisense
Ang-1	ACTGT GCAGA TGTAT ATCAA GC	GTGGA ATCTG TCATA CTGTG AA
Ang-2	GGAAG ACAAG CACAT CATCC	AGTAA GCCTG ATTCC CTTCC
Tie2	TCTGT GCTGT TCCTT CTTGC	CTTGA GTAAC TTCCA GCGGA
VEGF	AGCTA CTGCC ATCCA ATCGC	GGGCG AATCC AATTC CAAGA G
G3PDH	GTCAA CGGAT TTGGT CTGTA TT	AGTCT TCTGG GTGGC AGTGA T

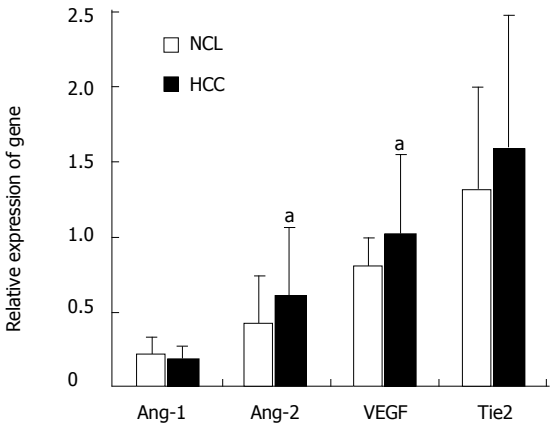

Figure 1 Expression of Ang-1, Ang-2, Tie2 and VEGF in HCC and NCL. HCC: hepatocellular carcinoma; NCL: noncancerous liver tissue. $^aP < 0.05$ *vs* NCL.

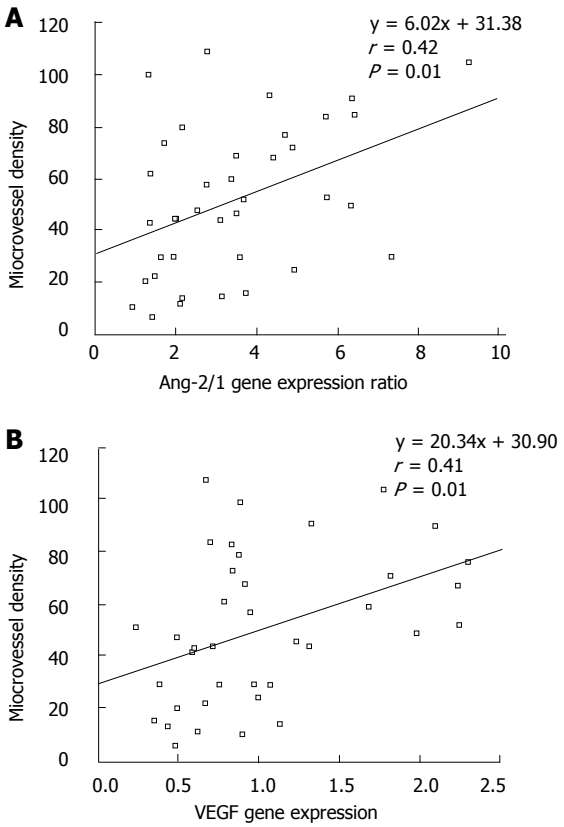

Figure 2 Relationship between Ang-2/Ang-1 ratio and microvessel density (**A**) and between vascular endothelial growth factor (VEGF) and microvessel density (**B**).

software and relative quantity according to standard reaction curve (R_v) was calculated according to the formula $R_v = R_{Gene}/R_{G3PDH}$ by computer.

Immunohistochemical assessment of vessel density

Paraffin-embedded tissues were sectioned (4 μm). The slides were deparaffined as usual, washed with tris buffered saline (TBS), and then incubated with 10% normal goat serum (Zhongshan Bio. CA). The sections were incubated with appropriately diluted (1:10) rat anti-human CD34 monoclonal antibody (Santa Cruz Biotechnology, CA) for 24 h at 4℃. Primary antibody was removed and washed with TBS, goat-anti-rat IgG peroxidase (Zhongshan Bio. CA) was then added. Finally the slices were stained as usual with haematoxilin and washed with distilled water. Quantification of blood vessels was carried out as previously described[18]. Any brown-stained endothelial cell cluster distinct from adjacent microvessels, tumor cells, or other stromal cells was considered as a single countable microvessel. The most vascular areas of tumors were identified on a low-power field (× 100), and vessels were counted in five high-power fields (× 200). The data were presented as mean ± SD. The process was performed by special pathologists in a blind manner.

Statistical analysis

Data were analyzed for significance with unpaired *t* test and ANOVA test. Statistical software SPSS 11.5 was used in the analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Ang-1, Ang-2, Tie2 and VEGF in NCL and HCC

The expression of Ang-2 and VEGF mRNA was significantly higher in HCC than in the NCL tissue ($P < 0.05$), but the Ang-1 and Tie2 mRNA showed no statistical significance ($P > 0.05$), though slightly lower level of Ang-1 in HCC was observed (Figure 1).

Correlation between Ang-2/Ang-1 ratio, VEGF and MVD

The expression of CD34 in NCL tissue was poor or even negative, but strong in HCC. Microvessel counting revealed that MVD was higher in HCC than in NCL. Because the balance between Ang-1 and Ang-2 mainly defines Tie2 signal, we determined the ratio of Ang-2 to Ang-1 and evaluated its correlation with MVD. VEGF was also involved. The Ang-2/Ang-1 ratio in HCC was positively related to MVD ($P = 0.01$, $r = 0.42$) and VEGF ($P = 0.01$, $r = 0.41$) (Figure 2).

Relationship between gene expression pattern and clinicopathological parameters

The values of Ang-1, Ang-2, Tie2, VEGF and Ang-2/Ang-1 ratio were classified according to HCC clinicopathological parameters including tumor size, tumor capsule, portal vein invasion, distal metastasis and histological

Table 3 Relation between gene expression pattern and clinicopathological parameters (mean ± SD)

Clinical parameters	Ang-2	Ang-1	Ang-2/1	Tie2	VEGF
Tumor size (cm)					
< 5 (n = 10)	0.267 ± 0.113	0.148 ± 0.089	2.175 ± 1.025	1.686 ± 0.550	0.552 ± 0.205
⩾ 5 (n = 28)	0.748 ± 0.422	0.212 ± 0.082	3.880 ± 2.067	1.570 ± 0.990	1.213 ± 0.537
Histlogical grade					
G1 (n = 12)	0.509 ± 0.320	0.168 ± 0.064	3.106 ± 1.409	1.511 ± 0.574	0.916 ± 0.497
G2 (n = 16)	0.633 ± 0.395	0.194 ± 0.081	3.543 ± 1.925	1.470 ± 0.726	1.095 ± 0.604
G3 (n = 10)	0.737 ± 0.512	0.234 ± 0.101	3.645 ± 2.470	1.921 ± 0.138	1.097 ± 0.585
Portal vein invasion					
positive (n = 11)	1.152 ± 0.376	0.232 ± 0.053	5.096 ± 1.691	1.880 ± 1.494	1.791 ± 0.423
negative (n = 27)	0.405 ± 0.163	0.180 ± 0.092	2.753 ± 1.631	1.488 ± 0.500	0.732 ± 0.254
Metastasis					
positive (n = 6)	1.320 ± 0.366	0.235 ± 0.061	5.912 ± 1.847	2.262 ± 1.734	2.007 ± 0.214
negative (n = 32)	0.490 ± 0.271	0.187 ± 0.088	2.967 ± 1.600	1.148 ± 0.619	0.857 ± 0.415
Tumor capsule					
positive (n = 13)	0.308 ± 0.135	0.178 ± 0.112	2.078 ± 0.887	1.733 ± 0.424	0.579 ± 0.198
negative (n = 25)	0.783 ± 0.429	0.203 ± 0.068	4.135 ± 1.998	1.529 ± 1.056	1.278 ± 0.558

grades. Ang-2, VEGF and Ang-2/Ang-1 ratio were all associated with the above clinicopathological parameters ($P < 0.05$) except for histological grades ($P > 0.05$), whereas Ang-1 and Tie2 showed no relation with any of the clinicopathological parameters ($P > 0.05$) (Table 3).

DISSCUSSION

VEGF and angiopoietin are two of the most important regulators for neovascularization. The former is the most potent angiogenic factor that promotes endothelial proliferation and increases vascular permeability by binding to its specific receptors in endothelial cells such as Flt-1, KDR/Flk-1 and Flt-4[19]. Animal models and *in vitro* experiments have shown that Ang-1, Ang-2 and Tie2 in association with VEGF constitute a system that regulates vascular quiescence and endothelial plasticity, through which a balanced state of vascular maturity and development of complex vascular networks can be achieved[20]. In the current study we investigated the expression of Ang-1, Ang-2, Tie2 and VEGF mRNA in HCC and noncancerous liver in an attempt to definite their exact role in carcinogenesis and progression of HCC *via* angiogenesis. To avoid possible influence of indefinite borderline carcinoma invasion, the noncancerous liver tissue was obtained from HCC position as far as possible during surgery. Ang-2, as an actively angiogenic factor in the presence of VEGF, is little expressed in physiological condition but highly expressed in several actively neovascularized organs such as endometrium, ovary, placenta *etc*[9,20-22]. This factor is up-regulated in many carcinomas, such as gastric, ovarian, colorectal and breast cancer, *etc*[23-26]. In this study, Ang-2 mRNA was significantly up-regulated in HCC compared with the nontumorous liver tissue, which is consistent with previous reports[2,27] and has been regarded as a contributor to recurrence, metastasis and poor prognosis of HCC. Besides, high expression of Ang-2 and low expression Ang-1 were found in HCC in comparison with NCL, indicating that they play a key role in the carcinogenesis and progression of HCC via angiogenesis. Tumorous angiogenesis is very different from the physiological process[28]. During this procession, vascular quiescence and stabilization are mediated by Ang-1, Ang-2 and Tie2 system. Therefore, the pathologic state of imbalanced Ang-2/Ang-1 ratio in the presence of VEGF plays a critical role in the transformation of noncancerous liver to liver cancer by initiating early neovascularization. Vajkoczy *et al*[29] reported that tumors in their very early stage are initiated by host vessels via VEGF, VEGF receptor-2 and Ang-2. Wong *et al*[16] also reported that angiopoietins, tie2 and VEGF are differentially expressed in the transformation of normal lung to non-small cell lung carcinomas.

CD34 protein, an endothelial-specific marker, was immunohistochemically stained and MVD was determined in HCC in our study. Ang-2/Ang-1 ratio was calculated and statistical analysis showed that Ang-2/1 ratio was positively correlated to MVD, and VEGF, but such relation was not found between Ang-1, Tie2 and MVD. Ang-2/Ang-1 ratio, Ang-2 and VEGF are all associated with tumor's clinicopathological characteristics such as tumor size, tumor capsule, portal vein invasion and metastasis, indicating that VEGF and angiopoiein/Tie2 system contributes greatly to the progress of HCC by modulating angiogenesis. Continuous growth of carcinoma induces hypoxia and necrosis in central portion, thus reversely up-regulating VEGF[30]. In the presence of Ang-2, tumor and invasion angiogenesis are greatly accelerated. A study[31] on C6 glioma showed that Ang-2 expresses strongly whereas VEGF expresses poorly when the tumor reaches 1mm in diameter, and when the tumor reaches more than 2 mm in diameter necrosis occurs in the central portion, both Ang-2 and VEGF are strongly expressed which induces intensive angiogenesis. Interestingly, in the current study Ang-1, Ang-2, Tie2, VEGF and Ang-2/Ang-1 ratio were all not statistically associated with histological grades of HCC, which is similar to a previous report on ovarian cancer[32].

In conclusion, expression of Ang-2 against Ang-1 through the Tie2 receptor in the presence of VEGF plays a critical role in initiating early neovascularization and in-

duces transformation of noncancerous liver to HCC. The consequently constant immature neovacularization in HCC further promotes angiogenesis and progression of tumors.

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REFERENCES

1 **Pisani P**, Parkin DM, Bray F, Ferlay J. Erratum: Estimates of the worldwide mortality from 25 cancers in 1990. Int. J. Cancer, 83, 18-29 (1999). *Int J Cancer* 1999; **83**: 870-873
2 **Poon RT**, Ng IO, Lau C, Yu WC, Fan ST, Wong J. Correlation of serum basic fibroblast growth factor levels with clinicopathologic features and postoperative recurrence in hepatocellular carcinoma. *Am J Surg* 2001; **182**: 298-304
3 **Poon RT**, Lau CP, Ho JW, Yu WC, Fan ST, Wong J. Tissue factor expression correlates with tumor angiogenesis and invasiveness in human hepatocellular carcinoma. *Clin Cancer Res* 2003; **9**: 5339-5345
4 **Carr BI**. Hepatocellular carcinoma: current management and future trends. *Gastroenterology* 2004; **127**: S218-S224
5 **Zhang ZL**, Liu ZS, Sun Q. Effects of thalidomide on angiogenesis and tumor growth and metastasis of human hepatocellular carcinoma in nude mice. *World J Gastroenterol* 2005; **11**: 216-220
6 **Folkman J**. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002; **29**: 15-18
7 **Pham VN**, Roman BL, Weinstein BM. Isolation and expression analysis of three zebrafish angiopoietin genes. *Dev Dyn* 2001; **221**: 470-474
8 **Davis S**, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 1996; **87**: 1161-1169
9 **Hata K**, Udagawa J, Fujiwaki R, Nakayama K, Otani H, Miyazaki K. Expression of angiopoietin-1, angiopoietin-2, and Tie2 genes in normal ovary with corpus luteum and in ovarian cancer. *Oncology* 2002; **62**: 340-348
10 **Mitsuhashi N**, Shimizu H, Ohtsuka M, Wakabayashi Y, Ito H, Kimura F, Yoshidome H, Kato A, Nukui Y, Miyazaki M. Angiopoietins and Tie-2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. *Hepatology* 2003; **37**: 1105-1113
11 **Sugimachi K**, Tanaka S, Taguchi K, Aishima S, Shimada M, Tsuneyoshi M. Angiopoietin switching regulates angiogenesis and progression of human hepatocellular carcinoma. *J Clin Pathol* 2003; **56**: 854-860
12 **Suri C**, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 1996; **87**: 1171-1180
13 **Maisonpierre PC**, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 1997; **277**: 55-60
14 **Teichert-Kuliszewska K**, Maisonpierre PC, Jones N, Campbell AI, Master Z, Bendeck MP, Alitalo K, Dumont DJ, Yancopoulos GD, Stewart DJ. Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc Res* 2001; **49**: 659-670
15 **Glade Bender J**, Cooney EM, Kandel JJ, Yamashiro DJ. Vascular remodeling and clinical resistance to antiangiogenic cancer therapy. *Drug Resist Updat* 2004; **7**: 289-300
16 **Wong MP**, Chan SY, Fu KH, Leung SY, Cheung N, Yuen ST, Chung LP. The angiopoietins, tie2 and vascular endothelial growth factor are differentially expressed in the transformation of normal lung to non-small cell lung carcinomas. *Lung Cancer* 2000; **29**: 11-22
17 **Torimura T**, Ueno T, Kin M, Harada R, Taniguchi E, Nakamura T, Sakata R, Hashimoto O, Sakamoto M, Kumashiro R, Sata M, Nakashima O, Yano H, Kojiro M. Overexpression of angiopoietin-1 and angiopoietin-2 in hepatocellular carcinoma. *J Hepatol* 2004; **40**: 799-807
18 **Weidner N**, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1-8
19 **Yamaguchi R**, Yano H, Nakashima Y, Ogasawara S, Higaki K, Akiba J, Hicklin DJ, Kojiro M. Expression and localization of vascular endothelial growth factor receptors in human hepatocellular carcinoma and non-HCC tissues. *Oncol Rep* 2000; **7**: 725-729
20 **Patan S**. Vasculogenesis and angiogenesis. *Cancer Treat Res* 2004; **117**: 3-32
21 **Hirchenhain J**, Huse I, Hess A, Bielfeld P, De Bruyne F, Krüssel JS. Differential expression of angiopoietins 1 and 2 and their receptor Tie-2 in human endometrium. *Mol Hum Reprod* 2003; **9**: 663-669
22 **Leach L**, Babawale MO, Anderson M, Lammiman M. Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta. *J Vasc Res* 2002; **39**: 246-259
23 **Ochiumi T**, Tanaka S, Oka S, Hiyama T, Ito M, Kitadai Y, Haruma K, Chayama K. Clinical significance of angiopoietin-2 expression at the deepest invasive tumor site of advanced colorectal carcinoma. *Int J Oncol* 2004; **24**: 539-547
24 **Sun XD**, Liu XE, Wu JM, Cai XJ, Mou YP, Li JD. Expression and significance of angiopoietin-2 in gastric cancer. *World J Gastroenterol* 2004; **10**: 1382-1385
25 **Zhang L**, Yang N, Park JW, Katsaros D, Fracchioli S, Cao G, O'Brien-Jenkins A, Randall TC, Rubin SC, Coukos G. Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. *Cancer Res* 2003; **63**: 3403-3412
26 **Sfiligoi C**, de Luca A, Cascone I, Sorbello V, Fuso L, Ponzone R, Biglia N, Audero E, Arisio R, Bussolino F, Sismondi P, De Bortoli M. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. *Int J Cancer* 2003; **103**: 466-474
27 **Deli G**, Jin CH, Mu R, Yang S, Liang Y, Chen D, Makuuchi M. Immunohistochemical assessment of angiogenesis in hepatocellular carcinoma and surrounding cirrhotic liver tissues. *World J Gastroenterol* 2005; **11**: 960-963
28 **Papetti M**, Herman IM. Mechanisms of normal and tumor-derived angiogenesis. *Am J Physiol Cell Physiol* 2002; **282**: C947-C970
29 **Vajkoczy P**, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A, Tonn JC, Menger MD, Breier G. Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J Clin Invest* 2002; **109**: 777-785
30 **Huang GW**, Yang LY, Lu WQ. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival. *World J Gastroenterol* 2005; **11**: 1705-1708
31 **Holash J**, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 1999; **18**: 5356-5362
32 **Hata K**, Nakayama K, Fujiwaki R, Katabuchi H, Okamura H, Miyazaki K. Expression of the angopoietin-1, angopoietin-2, Tie2, and vascular endothelial growth factor gene in epithelial ovarian cancer. *Gynecol Oncol* 2004; **93**: 215-222

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
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RAPID COMMUNICATION

Genetic instability of BRCA1 gene at locus D17S855 is related to clinicopathological behaviors of gastric cancer from Chinese population

Xue-Rong Chen, Wei-Zhong Zhang, Xing-Qiu Lin, Jin-Wei Wang

Xue-Rong Chen, Wei-Zhong Zhang, Jin-Wei Wang, Department of Surgery, Taizhou First People's Hospital, Huangyan 318020, Zhejiang Province, China
Xing-Qiu Lin, Institute of Cell Biology, Zhejiang University, Hangzhou 310031, Zhejiang Province, China
Co-first-author: Wei-Zhong Zhang
Correspondence to: Dr. Xue-Rong Chen, Department of Surgery, Taizhou First People's Hospital, Huangyan 318020, Zhejiang Province, China. zhangweizhong11@hotmail.com
Telephone: +86-576-4016999 Fax: +86-576-4016789
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Abstract

AIM: To investigate genetic instability of gene BRCA1 at locus D17S855, and their relationship with clinicopathological characteristics of gastric cancer in Chinese population.

METHODS: Microsatellite instability (MSI) and loss of heterozygosity (LOH) of gene BRCA1 at locus D17S855 were compared between 37 samples of gastric cancer and corresponding non-cancerous gastric tissue.

RESULTS: MSI at locus D17S855 was positive in 7 of 37 samples of gastric cancer (18.95%). MSI had a close relationship with TNM staging but no relation with lymph node metastasis, histological type or tumor differentiation. MSI positive frequency in TNM I + II (31.58%, 6/19) was much higher than that in TNM III + IV (5.56%, 1/18), ($P < 0.05$). LOH positive rate was 18.92% (7/37). LOH had no relationship to histological type, tumor differentiation or lymph node metastasis, but LOH positive rate in TNM III + IV was 33.33% (6/18), much higher than that in TNM I + II (5.26%, 1/19), ($P < 0.05$). BRCA1 protein was expressed in 14 of 37 samples of gastric cancer. The positive rates of BRCA1 protein in TNM I + II and TNM III + IV were 57.89% and 16.67%, respectively, ($P < 0.05$). The positive rate of BRCA1 protein was 77.78% in high differentiation samples, 30.77% in middle differentiation and 12.50% in lower differentiation samples, ($P < 0.05$).

CONCLUSION: MSI of BRCA1 gene could be used as a molecular marker in early phases of sporadic gastric cancer in Chinese population. LOH occurs at later period of gastric cancer, therefore, it could be used as prognostic factor.

INTRODUCTION

Gastric cancer is one of the most common cancers worldwide, and growing evidence suggests that accumulation of multiple alterations such as activation of proto-oncogenes and inactivation of tumor suppressor genes is responsible for the development and progression of gastric cancer. Genetic instability of oncogenes such as microsatellite instability (MSI) and loss of heterozygosity (LOH) is probably associated with mutations in genes responsible for tumor-genesis, and they play important roles in tumor clinical pathology[1-4]. The studies of MSI and LOH of gastric cancer have been focused on genetic instability of P53, P16 and FHIT, but studies of gene BRCA1 gene are very few. The gene BRCA1 is located in the chromosomal region 17q21. Many studies have reported that BRCA1 is a tumor suppressor gene of breast and ovarian tumors, but only few studies have been done on Chinese gastric cancer[5-7]. The present study was undertaken to investigate MSI and LOH of gene BRCA1 at locus D17S855 in Chinese gastric cancer, their influence on the expression of BRCA1 protein, and their relationship with clinical pathological characteristics of gastric cancer.

MATERIALS AND METHODS

Tumor samples and DNA extraction

A total of 37 formalin-fixed, paraffin-embedded tumor samples and corresponding non-tumor samples from the same sporadic gastric patients were collected. These samples included 30 cases of tubular adenocarcinoma and 7 cases of mucoid adenocarcinoma. All the samples were sectioned into 10 μm sections, and tumor samples contained at least 90% tumor cells. Genomic DNAs

from tumor and normal components were extracted by proteinase K and phenol-chloroform extraction methods.

Polymerase Chain Reaction (PCR)

Primers of D17S855 locus were: 5'-GGA TGG CCT TTT AGA AAG TGG- 3' and 5'-ACA CAG ACT TGT CCT ACT GCC -3' (Bioasia Shanghai)[8]. Polymerase chain reaction (PCR) was carried out in 50 μL reaction mixtures containing 1.0 mmol/L $MgCl_2$, 10 × Buffer 5 μL, 200 μmol/L dNTP, Taq polymerase 2units and primers 50 *p*mol/L. PCR was carried out for one cycle of 94℃ for 4 min followed by 35 cycles of 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min, followed by a final extension for 10 min at 72℃. The PCR productions were detected by 2% agarose gel containing ethidium bromide, underwent electrophoresis for 30 min at 15 V/cm, and were examined under ultraviolet light.

Single strand conformation polymorphism analysis of MSI and LOH

The PCR production and an equal volume of denaturing stop solution (98% deionised formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) were heated for 10 min and then were rapidly cooled on ice. Electrophoresis was carried out on 8% polyacrylamide gels at 100 V for 4 h. After electrophoresis, the gels were ordinarily silver-stained. To detect MSI and LOH, the PCR productions of normal and tumor DNA from the same patients were run in adjacent lanes. MSI was positive as either tumor samples added an allele band or moved as compared with normal tissue. LOH was positive as tumor samples lacked an allele band as compared with normal tissue.

Immunohistochemistry staining

Immunohistochemistry for BRCA1 protein was performed on 5 μm sections from paraffin-embedded tumor tissue which was dewaxed in xylene and dehydrated in a graded ethanol series. The sections were immersed in 3% hydrogen peroxidase for 10 min to block endogenous peroxidase activity, and then rinsed in water. Thereafter, the sections were heated in a microwave for 5 min at 100℃ to retrieve antigen. The sections were incubated with primary antibody BRCA1 (Santa Cruz Biotechnology, USA), then the sections were allowed to react by standard Envision method using secondary antibody (DAKO, Denmark). The binding was visualized by DAB, and then the samples were counterstained with hematoxylin.

Imaging analysis and data collection

After immunohistochemistry staining, sections were analyzed by Leica-Qwin computer imaging techniques. We selected twenty continuous high microscopical views that did not overlap. Then we tested the gray-value of background and named as GA. The gray-value of BRCA1 positive granules was named as Ga and area density of BRCA1 positive cells in the view area was named as A_{Aa}. We used Excel function to compute the value of PU (positive unit) which represented expression intensity of BRCA1 protein in gastric cancer cell. The highest gray value was 255 in Leica-Qwin system.

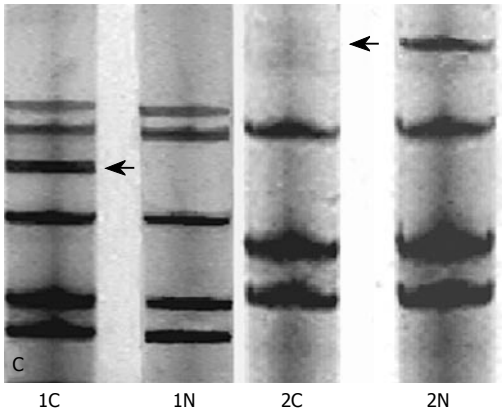

Figure 1 Polyacrylamide gel electrophoresis (PAGE) of BRCA1 gene at D17S855 locus Compared with normal tissue (1N), MSI was positive as added an allele band (↑) in tumor tissue (1C). Compared with normal tissue (2N), LOH was positive as lacked an allele band (↑) in tumor tissue (2C).

Statistical analysis

Statistical analysis was performed using analysis of variance (AVONA) and *t*- test. A P value of < 0.05 was considered significant difference.

$$PU = \frac{G_a - G_A}{(1 - A_{Aa}) \times 255} \times 100$$

RESULTS

Single strand conformation polymorphism analysis (PCR-SSCP) of MSI and LOH

In our experiment, 37 paired normal/tumor DNAs were successfully amplified by PCR method, and were tested by 8% polyacrylamide gels. MSI was positive as added an allele band in tumor tissue as compared with normal tissue; LOH was positive as lacked an allele band in tumor tissue as compared with normal tissue (Figure 1).

Of locus D17S855 at gene BRCA1, the positive frequency of MSI in 37 cases of gastric cancer was 18.92% (7/37) (Table 1). MSI of BRCA1 gene was significantly correlated with clinical TNM staging and degree of tumor differentiation; however, there was no significant difference between MSI-positive and MSI-negative cases in tumor histological type or lymph node metastasis. In tumor node metastasis (TNM) staging, the positive frequency of MSI in stage TNM I + II (31.58%) was more than that in stage TNM III + IV (5.56, $P < 0.05$), and the frequency of LOH-positive of 37 cases of gastric cancer was 18.92% (7/37) (Table 2). LOH of BRCA1 gene was significantly correlated with clinical TNM staging; however, there was no significant difference between LOH-positive and LOH-negative cases in tumor histological type, tumor differentiation degree or lymph node metastasis. In tumor node metastasis (TNM) staging, the positive frequency of LOH in stage TNM III + IV (33.33%) was higher than that in stage TNM I + II (5.26%, $P < 0.05$).

Table 1 Relationship between hereditary instability of BRCA1 gene at locus D17S855 and clinicopathological parameters of gastric cancer

Clinicopathological parameters	Case (*n*)	MSI positive rates (%)	LOH positive rates (%)	BRCA1 positive rates (%)	BRCA1 expression (PU Mean±S)
Histology	37	7 (18.92%)	7 (18.92%)	14 (37.84%)	33.56 ± 2.23
Tubuladenoma	30	6 (20.00%)	6 (20.00%)	12 (40.00%)	32.87 ± 2.93
High differentiation	9	4 (44.44%) 1 (7.69%)	1 (11.11%)	7 (77.78%)[a]	34.03 ± 1.79
Middle differentiation	13	1 (12 .50 %)	1 (7.69%)	4 (30.77%)	33.49 ± 2.34
Low differentiation	8	1 (14.29%)	4 (50.00%)	1 (12.50%)	33.58 ± 1.67
Mucoadenoma	7		1 (14.29%)	2 (28.57%)	32.87 ± 2.59
Lymph metastasis					
No	17	5 (29.41%)	1 (5.88%)	9 (52.94%)	34.75 ± 1.78
Yes	20	2 (10.00%)	6 (30.00%)	5 (25.00%)	33.14 ± 2.14
TNM staging					
Stage I + II	19	6 (31.58%)	1 (5.26%)	11 (57.89%)	33.88 ± 2.38
Stage III + IV	18	1 (5.56%)[a]	6 (33.33%)[a]	3 (16.67%)[a]	33.23 ± 2.34

[a]$P < 0.05$.

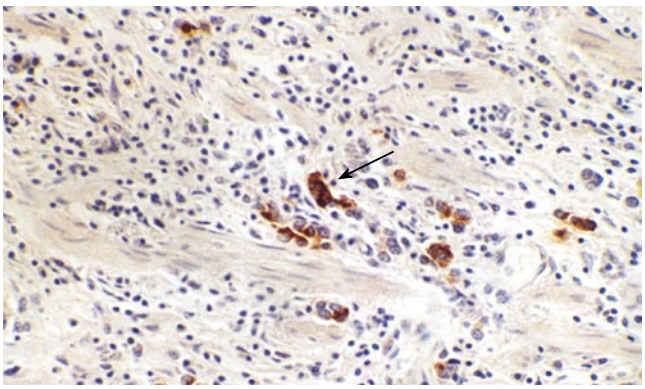

Figure 2 The expression of BRCA1 protein in gastric cancer. The brown-yellow granules of BRCA1 protein were mostly located in nucleolus. The cytoplasm and membrane of cells were also stained (HE stained↑). ×100.

Immunohistochemistry analysis of BRCA1 protein

The expression of BRCA1 protein in gastric cancer was the brown-yellow granules, mostly located in nucleolus, and cytoplasm and membrane of cells were also stained (Figure 2). The frequency of BRCA1 protein-positive in 37 cases of gastric cancer was 37.84% (14/37) (Table 2). Expression of BRCA1 protein was significantly correlated with clinical TNM staging and tumor differentiation degree; however, expression of BRCA1 protein was not associated with tumor histological type or lymph node metastasis. The positive frequency of BRCA1 protein in TNM I + II (57.89%) was much higher than TNM III + IV stage (16.67%, $P < 0.05$), and BRCA1 positive-rate in well differentiation cases was higher than poor differentiation cases. The frequency of BRCA1 protein-positive decreased as tumor differentiation went down, 77.78% in high differentiation cases, 30.77% in middle differentiation cases, and 12.50% in low differentiation cases ($P < 0.05$). There was no difference in BRCA1 protein expression intensity analyzed by computer imaging.

Table 2 Relationship between MSI, LOH of BRCA1 gene at locus D17S855 and BRCA1 protein expression

Grouping	Cases	BRCA1 protein positive rates	BRCA1 expression (PU Mean±S)
MSI positive group	7	85.71% (6/7)	34.05 ± 1.91
MSI negative group	30	26.67% (8/30)[a]	32.59 ± 2.23
LOH positive group	7	28.57% (2/7)	32.87 ± 2.35
LOH negative group	30	40.00% (12/30)	34.03 ± 2.66

[a]$P < 0.05$.

DISCUSSION

In 1994, the breast-cancer susceptibility gene, BRCA1, was identified by positional cloning; subsequently, this gene has been the subject of intensive research effort. BRCA1, located on chromosome 17q21 and encoding a tumor suppressor gene that functions, in part, as a caretaker gene in preserving chromosomal stability, is composed of 22 coding exons distributed over 100 kb of genomic DNA[3]. This gene encodes 1863 amino acids, and more than 200 different germline mutations associated with cancer susceptibility have been identified. Cell cycle checkpoints play an essential role in cell survival by preventing the propagation of DNA damage through cell cycle progression before DNA repair. Recent studies have demonstrated that both ATM and BRCA1 are required for effective S-phase and G2/M-phase checkpoints. Other work has indicated that BRCA1 regulates G2/M DNA damage induced checkpoints through its ability to activate Chk1 kinase and thereby induce signaling cascades downstream of Chk1. BRCA1 functions as a co-activator of p53-mediated gene transcription. Other studies have shown that overexpression of BRCA1 results in the transcriptional activation of GADD45 in a p53-dependent manner. As GADD45 has been implicated in G2/M checkpoints, BRCA1 may in part activate G2/M checkpoints by induction of GADD45 protein[3,9,10].

The association of the BRCA1 gene with susceptibility to breast and ovarian cancer has been strongly demonstrated, and locus D17S855 was found as one of the best candidate loci to detect tumor suppressor genes[10]. Mori *et al* found that BRCA1 might play an important role in the development of esophagus cancer with high frequent LOH[9]. Therefore, locus D17S855 was used in this experiment to detect MSI and LOH in gastric cancer of Chinese population.

In this study, the frequency of MSI-positive in 37 cases of gastric cancer was 18.92%. MSI of BRCA1 gene was significantly correlated with clinical TNM staging and tumor differentiation degree; however, there was no significant difference between MSI-positive and MSI-negative cases in tumor histological type or lymph node metastasis. TNM staging represents the prognosis of tumor, and we found in the experiment that the frequency

of MSI in stage TNM I+ II was more than that in stage TNM III + IV. Therefore, as far as gene BRCA1 is concerned, high frequency of MSI endowed with a good prognosis. Furthermore, the positive frequency of MSI decreased as tumor differentiation went down, tumor differentiation correlated with the development and malignant degree of tumor. According to the experiment results, MSI of BRCA1 was an early period molecule marker of sporadic gastric cancer. Candusso *et al* found that LOH often occurred in the later stage of cancer, mostly with lymph metastasis[10]. The positive frequency of LOH in 37 cases of gastric cancer in our study was 18.92%, and the positive frequency of LOH in stage TNM III + IV was higher than that in stage TNM I + II. The results suggested that LOH of BRCA1 gene might occur in the later stage of gastric cancer and could be a marker that predicts prognosis of gastric cancer.

The positive frequency of BRCA1 protein in MSI positive group was higher than MSI negative group, but there was no significant difference between LOH positive and negative groups. Expression of BRCA1 protein was significantly correlated with clinical TNM staging and tumor differentiation degree; however, expression of BRCA1 protein was not associated with tumor histological type or lymph node metastasis. The frequency of BRCA1 protein-positive decreased as tumor differentiation went down. All these data suggested that BRCA1 gene might restrain gastric cancer from developing further.

In conclusion, MSI of BRCA1 is an early event in development of sporadic gastric cancer in Chinese population and LOH of BRCA1 gene occurs in later stage, therefore BRCA1 gene might be a suppressor gene for gastric cancer.

REFERENCES

1 **Jackson AL**, Loeb LA. Microsatellite instability induced by hydrogen peroxide in Escherichia coli. *Mutat Res* 2000; **447**: 187-198

2 **Sprecher CJ**, Puers C, Lins AM, Schumm JW. General approach to analysis of polymorphic short tandem repeat loci. *Biotechniques* 1996; **20**: 266-276

3 **Yoshida K**, Miki Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci* 2004; **95**: 866-871

4 **Rouba A**, Kaisi N, Al-Chaty E, Badin R, Pals G, Young C, Worsham MJ. Patterns of allelic loss at the BRCA1 locus in Arabic women with breast cancer. *Int J Mol Med* 2000; **6**: 565-569

5 **Barnetson R**, Jass J, Tse R, Eckstein R, Robinson B, Schnitzler M. Mutations associated with microsatellite unstable colorectal carcinomas exhibit widespread intratumoral heterogeneity. *Genes Chromosomes Cancer* 2000; **29**: 130-136

6 **Huiping C**, Kristjansdottir S, Bergthorsson JT, Jonasson JG, Magnusson J, Egilsson V, Ingvarsson S. High frequency of LOH, MSI and abnormal expression of FHIT in gastric cancer. *Eur J Cancer* 2002; **38**: 728-735

7 **Berney CR**, Fisher RJ, Yang J, Russell PJ, Crowe PJ. Genomic alterations (LOH, MI) on chromosome 17q21-23 and prognosis of sporadic colorectal cancer. *Int J Cancer* 2000; **89**: 1-7

8 **Garcia-Patiño E**, Gomendio B, Lleonart M, Silva JM, Garcia JM, Provencio M, Cubedo R, España P, Ramón y Cajal S, Bonilla F. Loss of heterozygosity in the region including the BRCA1 gene on 17q in colon cancer. *Cancer Genet Cytogenet* 1998; **104**: 119-123

9 **Mori T**, Aoki T, Matsubara T, Iida F, Du X, Nishihira T, Mori S, Nakamura Y. Frequent loss of heterozygosity in the region including BRCA1 on chromosome 17q in squamous cell carcinomas of the esophagus. *Cancer Res* 1994; **54**: 1638-1640

10 **Candusso ME**, Luinetti O, Villani L, Alberizzi P, Klersy C, Fiocca R, Ranzani GN, Solcia E. Loss of heterozygosity at 18q21 region in gastric cancer involves a number of cancer-related genes and correlates with stage and histology, but lacks independent prognostic value. *J Pathol* 2002; **197**: 44-50

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PO Box 2345, Beijing 100023, China
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CASE REPORT

Multiple von Meyenburg complexes mimicking diffuse liver metastases from esophageal squamous cell carcinoma

Stefan Fritz, Thilo Hackert, Hendrik Blaker, Werner Hartwig, Lutz Schneider, Markus W Buchler, Jens Werner

Stefan Fritz, Thilo Hackert, Werner Hartwig, Lutz Schneider, Markus W Buchler, Jens Werner, Departments of General and Visceral Surgery, University of Heidelberg, Germany
Hendrik Blaker, Departments of Pathology, University of Heidelberg, Germany
Correspondence to: Jens Werner, MD, Departments of General and Visceral Surgery, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg,
Germany. jens.werner@med.uni-heidelberg.de
Telephone: +49-6221-566920 Fax: +49-6221-564863
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Abstract

Von Meyenburg complexes are benign liver lesions consisting of adenomatous bile duct proliferates. We present two patients suffering from esophageal cancer accompanied by the occurrence of von Meyenburg complexes. Preoperative computerized tomography (CT) of the liver had not shown these lesions. In one of the patients, diffuse nodular manifestation was found in both liver lobes, mimicking diffuse hepatic metastases. Intraoperative frozen section revealed the benign nature of the lesions in both cases. The patients underwent esophageal resection without complications. To the best of our knowledge, the coincidence of von Meyenburg complexes and esophageal cancer has never been reported before. This uncommon entity should be taken into consideration as a differential diagnosis of liver lesions in malignancies. It underlines the importance of intraoperative frozen section for liver lesions of unknown origin.

Key words: Von Meyenburg complexes; Bile duct hamartomas; Esophageal carcinoma; Liver metastasis

Fritz S, Hackert T, Blaker H, Hartwig W, Schneider L, Buchler MW, Werner J. Multiple von Meyenburg complexes mimicking diffuse liver metastases from esophageal squamous cell carcinoma. *World J Gastroenterol* 2006; 12(26): 4250-4252

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INTRODUCTION

Von Meyenburg complexes are benign liver lesions consisting of dilated bile duct structures with a surrounding fibrous stroma. Their incidence is age-dependent and they are observed about 1% in children and 5%-6% in adults[1]. Usually, these lesions do not have any pathological value and are incidental findings in asymptomatic patients[1]. However, a malignant transformation and association with cholangiocarcinomas have been discussed, but not yet proven[2,3]. As von Meyenburg complexes are small lesions, they are usually not detected on radiological examinations, including ultrasound, CT and magnetic resonance imaging (MRI)[4-7].

We present herein two cases of preoperatively undetected von Meyenburg complexes associated with primary esophageal cancer and review the present literature concerning these lesions.

CASE REPORT

Patient 1 is a 66-year-old male suffering from an esophageal squamous cell carcinoma. Preoperatively, distant metastases were excluded by abdominal ultrasound, chest X-ray and high resolution CT-imaging of the thorax and liver (Figure 1). Surgical therapy with abdomino-thoracic resection of the esophagus was indicated.

Intraoperatively, abdominal exploration revealed multiple white nodular lesions in both lobes of the liver. With a diameter of 2-3 mm, they had the macroscopic aspect of diffuse liver metastases (Figure 2). To confirm the diagnosis of metastases, which would have excluded the patient from further curative resection of the primary esophageal carcinoma, a frozen section was performed. Surprisingly, histology showed benign microhamartomas, so-called von Meyenburg complexes (Figure 3). Because of the benign nature of the lesions, esophageal resection was performed as planned. The postoperative course was uneventful, liver function was normal and the patient recovered quickly.

Patient 2 is a 47-year-old male also suffering from an esophageal squamous cell carcinoma. Comparable to patient 1, the preoperative liver CT scan showed no pathological findings except for liver steatosis and a small cyst in segment IV/VIII.

Intraoperatively, we additionally found a suspicious liver lesion of 5 mm size in segment III. In contrast to the case described above, this was a solitary lesion, with the macroscopic aspect of a metastasis. However, frozen section revealed a sclerotic von Meyenburg complex. Consequently, the operation was performed as planned. No further complications occurred in the postoperative course.

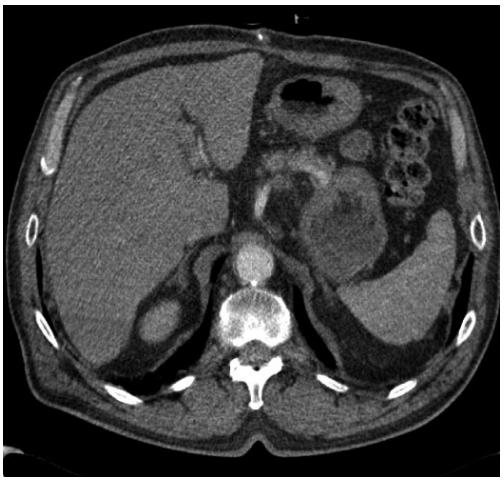

Figure 1 Preoperative CT scan (patient 1). No parenchymal inhomogeneity or focal lesions can be detected.

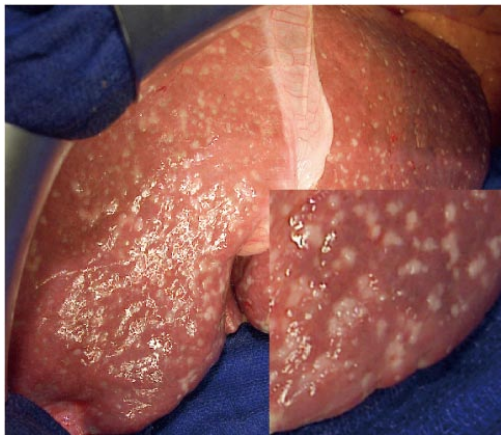

Figure 2 Intraoperative aspects (patient 1). Multiple small nodules are present in both lobes of the liver, mimicking diffuse metastatic lesions.

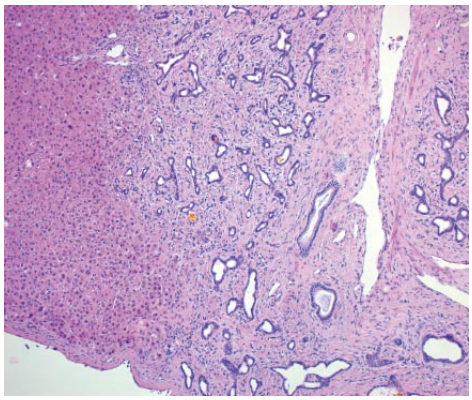

Figure 3 Histologic staining of a von Meyenburg complex (patient 1).

DISCUSSION

Esophageal cancer is an important cause of cancer death, with an incidence of about 8-10/100 000 and 13 300 deaths in the United States in 2004[8]. About 35% of all patients already suffer from liver metastasis by the time of diagnosis of the primary tumor. Since patients are excluded from potentially curative resection of the tumor when metastases are present, the diagnosis of liver metastases plays a crucial role for further treatment. The prognosis of these patients is also highly dependant on the respectability of the tumor.

Distant metastases, especially liver metastasis, can be diagnosed by CT scan or MRI with high sensitivity and specificity[6]. Sensitivities of these diagnostic means range between 74% and 85%[9]. In these series, almost all false-negative results occurred when lesions were less than 1.5 cm in diameter. Therefore, non-invasive detection of small metastases can be difficult or even impossible.

When suspicious lesions are found by CT scan, further differentiation is possible by additional MRI imaging[5]. Differential diagnosis of liver metastases includes benign liver lesions, including hemangiomas, adenomas, von Meyenburg complexes or infectious lesions e.g. miliary tuberculosis[5].

Bile duct hamartomas (von Meyenburg complexes) of the liver are usually detected during laparotomy or autopsy as an incidental finding. Multilocular occurrence is possible although they are rarely spread throughout the whole liver, as it was observed in our first patient. They may be found in normal liver tissue, but also in association with Caroli's syndrome, congenital hepatic fibrosis (CHF) or autosomal dominant polycystic kindney disease (ADPKD)[10]. Histology of von Meyenburg complexes consists of a variable number of dilated small bile ducts, embedded in a fibrous, sometimes hyalinizing stroma (Figure 3).

If detected by CT scan or MRI, von Meyenburg complexes appear as small intrahepatic cystoid lesions. The lesions are frequently located adjacent to the portal veins, although the lesions can also be located everywhere else[5]. However, it remains difficult to differentiate between metastases and benign liver lesions. Moreover, small liver lesions with a diameter of less than 1.5 cm are often not detected by CT or MRI[9].

Since the treatment of metastatic disease is completely different from resectable esophageal cancer, liver lesions need to be identified and characterized as early as possible. In our presented patients, the preoperative staging did not reveal any liver metastases. This underlines the importance of exact diagnostic measures in cases of unexpected intraoperative findings. Besides intraoperative ultrasound of the liver, frozen section is the gold standard for further differentiation of liver lesions of unknown origin.

Von Meyenburg complexes are defined as innocuous lesions. However, there are about 10 reported cases of neoplastic transformation of von Meyenburg complexes resulting in cholangiocarcinomas[2,3].

In conclusion, von Meyenburg complexes are an important differential diagnosis of hepatic metastases. As the existence of liver metastases is crucial for therapeutic decision making in malignant diseases, this differential diagnosis must be carefully clarified. Since von Meyenburg complexes are usually less than 5 mm in size, they can escape preoperative radiologic diagnostics. The macroscopic appearance of von Meyenburg complexes can mimic liver metastasis as demonstrated in our reported patients. This underlines the importance of intraoperative frozen sections to make the correct diagnosis.

REFERENCES

1 **Redston MS**, Wanless IR. The hepatic von Meyenburg complex: prevalence and association with hepatic and renal cysts among 2843 autopsies [corrected]. *Mod Pathol* 1996; **9**: 233-237

2 **Röcken C**, Pross M, Brucks U, Ridwelski K, Roessner A. Cholangiocarcinoma occurring in a liver with multiple bile duct hamartomas (von Meyenburg complexes). *Arch Pathol Lab Med* 2000; **124**: 1704-1706

3 **Jain D**, Sarode VR, Abdul-Karim FW, Homer R, Robert ME. Evidence for the neoplastic transformation of Von-Meyenburg complexes. *Am J Surg Pathol* 2000; **24**: 1131-1139

4 **Mortelé B**, Mortelé K, Seynaeve P, Vandevelde D, Kunnen M, Ros PR. Hepatic bile duct hamartomas (von Meyenburg Complexes): MR and MR cholangiography findings. J *Comput Assist Tomogr* 2002; **26**: 438-443

5 **Luo TY**, Itai Y, Eguchi N, Kurosaki Y, Onaya H, Ahmadi Y, Niitsu M, Tsunoda HS. Von Meyenburg complexes of the liver: imaging findings. *J Comput Assist Tomogr* 1998; **22**: 372-378

6 **Pokieser P**, Memarsadeghi M, Danzer M, Prokesch R, Partik B, Wenzl E. [Staging of carcinomas of the upper gastrointestinal tract. The current status of diagnostic imaging]. *Radiologe* 1999; **39**: 555-561

7 **Maher MM**, Dervan P, Keogh B, Murray JG. Bile duct hamartomas (von Meyenburg complexes): value of MR imaging in diagnosis. *Abdom Imaging* 1999; **24**: 171-173

8 **Jemal A**, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ. Cancer statistics, 2004. *CA Cancer J Clin* 2004; **54**: 8-29

9 **Haider MA**, Amitai MM, Rappaport DC, O'Malley ME, Hanbidge AE, Redston M, Lockwood GA, Gallinger S. Multidetector row helical CT in preoperative assessment of small (< or = 1.5 cm) liver metastases: is thinner collimation better? *Radiology* 2002; **225**: 137-142

10 **Leuven KU**, Desmet VJ. Pathogenesis of ductal plate malformation. *J Gastroenterol Hepatol* 2004; **19**: 356-360

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CASE REPORT

Systemic lupus erythematosus following virological response to peginterferon alfa-2b in a transplanted patient with chronic hepatitis C recurrence

Francesca Lodato, Maria Rosa Tamé, Antonio Colecchia, Chiara Racchini, Francesco Azzaroli, Antonia D'Errico, Silvia Casanova, Antonio Pinna, Enrico Roda, Giuseppe Mazzella

Francesca Lodato, Maria Rosa Tamé, Antonio Colecchia, Chiara Racchini, Francesco Azzaroli, Enrico Roda, Giuseppe Mazzella, Department of Internal Medicine and Gastroenterology, University of Bologna, Italy
Antonia D'Errico, Silvia Casanova, Department of Experimental Pathology, "F. Addari" Institute of Oncology, University of Bologna, Italy
Antonio Pinna, Department of Surgery and Transplantation, Sant' Orsola-Malpighi Hospital, University of Bologna, Italy
Correspondence to: Dr. Francesca Lodato, Dipartimento di Medicina Interna e Gastroenterologia, U.O. di Gastroenterologia, Via Massarenti 9, Bologna 40138, Italy. francesca.lodato@inwind.it
Telephone: +39-51-6363376 Fax: +39-51-6364120
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Abstract

Autoimmune manifestations are common both in patients chronically infected by hepatitis C virus, and in patients transplanted for non-autoimmune diseases. A correlation between interferon based treatment and autoimmune diseases or the development of autoantibodies is well established in non-transplanted patients, but few data are available about transplanted patients. It is unclear whether interferon may increase the incidence of acute cellular rejection and there are few reports on the development of atypical autoimmune manifestations during post-liver transplantation interferon or pegylated interferon treatment. We describe a case of systemic lupus erythematosus following treatment with pegylated interferon alfa-2b in a transplanted patient with recurrence of chronic hepatitis C. Our experience suggest that pegylated interferon may induce autoimmune diseases in the immunosuppressed host, different from acute cellular rejection and call for a great attention to possible autoimmune disorders development during interferon based treatments in liver transplanted patients.

Key words: Hepatitis C virus; Liver transplantation; Autoimmunity; Immunosuppression; Systemic lupus erythematosus

Lodato F, Tamè MR, Colecchia A, Racchini C, Azzaroli F, D' Errico A, Casanova S, Pinna A, Roda E, Mazzella G. Systemic lupus erythematosus following virological response to peginterferon alfa-2b in a transplanted patient with chronic hepatitis C recurrence. *World J Gastroenterol* 2006; 12(26): 4253-4255

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INTRODUCTION

Autoimmune manifestations are common in patients chronically infected by hepatitis C virus (HCV)[1]. On the other hand, tissue autoantibodies are common in liver recipients transplanted for non autoimmune diseases and may be associated with negative graft outcome[2,3]. The safety and efficacy of interferon (IFNs) and the newest pegylated interferons (Peg-IFNs) for the treatment of recurrent hepatitis C in transplanted patients are still debated[4,5]. In particular, it is unclear whether IFN may increase the incidence of acute cellular rejection (ACR) and there are no reports on the development of atypical autoimmune manifestations during post-liver transplantation (LT) IFN or Peg-IFN treatment.

We report a case of severe autoimmune disease, different from ACR, during treatment with Peg-IFN alfa-2b in a transplanted patient with recurrence of chronic hepatitis C (CHC).

CASE REPORT

A 55-year-old man, underwent LT in March 2001 for HCV genotype 1 liver related cirrhosis. Acute immunosuppressive (IS) schedule was cyclosporine, azathioprine (AZA) and steroids. According to the Transplantation Unit IS protocol, AZA and steroids were stopped 3 wk and 1 year after LT, respectively. Screening tests for LT revealed the presence of cryoglobulins with a cryocrite of 8% and antinuclear antibodies (ANA) at low titre (1/160) with homogeneous pattern. After LT, clinical outcome was regular until January 2002, when the patient showed a persistent mild increase of transaminases (ALT 115 U/L and AST 103 U/L) with high viral load (17.5 MEq/mL, Versant HCV-RNA 3.0 bDNA, Bayer). Liver histology showed mildly active chronic hepatitis with severe fibrosis, presence of lymphocytes and macrovescicular steatosis, sug-

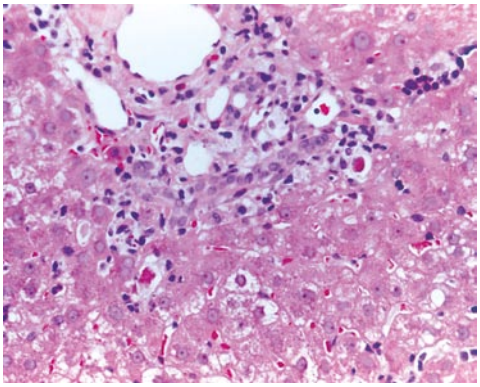

Figure 1 Liver histology before starting antiviral treatment.

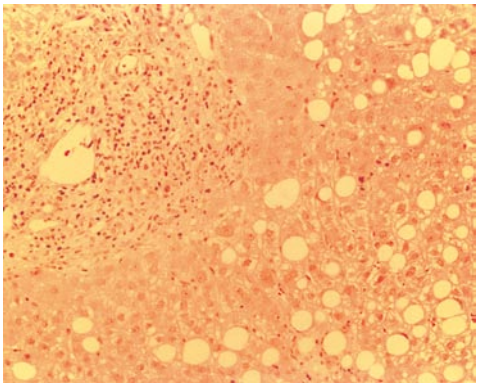

Figure 2 Liver histology after stopping antiviral treatment.

gestive of HCV recurrence (Figure 1).

In October 2002 the patient started a cycle of Peg-IFN alfa-2b (1.1 mcg/kg per week) and Ribavirin (6.4 mg/kg per day). After 4 wk of treatment transaminases were normal. HCV-RNA showed a 2 log fall (0.01 MEq/mL) at wk 12; became undetectable by branched DNA, but still positive by polymerase chain reaction (TMA test, Versant HCV-RNA, Bayer) at wk 24 and finally negative by PCR at wk 36.

At wk 44 the patient presented migrant arthritis and the following biochemical parameters: normal transaminases, CyA 240 ng/mL, increased gamma-glutamyltransferase (γGT), alkaline phosphatase (ALP) and bilirubin (384 U/L, 690 U/L and 1.69 mg/dL, respectively), gamma-globulins 30%, Waaler-Rose 1/1280, ANA 1/640 and anti-DNA positive. No vascular or biliary complications were revealed by ultrasound and computed tomography, nor any signs of infectious diseases were present. Suspicion of an immune mediated manifestation, prednisone 10 mg/d was started. However, despite the presence of signs of autoimmunity we decided to complete the Peg-IFN cycle in consideration of the fact that we were almost at the end of the planned 48 wk of treatment with the patient responding to Peg-IFN.

At wk 48 the patient was asymptomatic, transaminases and bilirubin were normal, HCV-RNA negative by PCR, while ALP and γGT were decreased (ALP 350 U/L and γGT 94 U/L). Peg-IFN was stopped and steroids were maintained.

One month later, the patient developed pleuro-pericardial effusion and ascites. Liver function tests (LFTs) were normal, HCV-RNA was negative (PCR) and CyA within the therapeutic range; ANA was very high (1/1280) as well as perinuclear anti-neutrophil cytoplasmatic antibodies (pANCA) (1:320). Therefore, our patient developed a syndrome characterised by high titre autoantibodies, migrant arthritis and serositis. Following the current criteria, the diagnosis of systemic lupus erythematosus could have been made[6,7]. Prednisone was increased to 15 mg/d and diuretics introduced, resulting in the remission of liquid effusions and a progressive reduction of autoantibodies (ANA 1:160 and pANCA negative). We did not increase further the prednisone dosage in consideration of the possibility of an HCV re-activation in an already immunosuppressed patient and because of the initial clinical remission of autoimmune manifestations.

In March 2004, four months later, the patient presented an important increase of all LFT (GOT 257 U/L, GPT 197 U/L, ALP 438 U/L, γGT 356 U/L, total bilirubin 20.35 mg/dL, with direct bilirubin 17.20 mg/dL) with normal eosinophils, serum HCV-RNA positive at low titre (2.96 MEq/mL). CyA was close to the lower limit of the therapeutic range (100 ng/mL). Liver biopsy revealed the presence of interface hepatitis, numerous rosettes, cholangitis with ductular proliferation and biliocytes regression (Figure 2). Histological findings were not suggestive for HCV recurrence and did not fulfil the standard criteria for acute or chronic rejection[8]. Therefore, International Criteria for Autoimmune Hepatitis (AIH score)[9] were applied, according to which the patient was categorized as positive for "probable AIH" (score + 11). Consequently, steroid treatment was increased and the patient was switched from cyclosporine to tacrolimus. Response to immunosuppressive treatment was slow but progressive until normalization of LFTs.

DISCUSSION

It is well known that treatment with IFN may cause autoimmune diseases or the development of autoantibodies in non transplanted patients[10-13] while few data are available on transplanted ones. In particular, to the best of our knowledge, it has never been described the development of SLE after LT during interferon treatment. Only two cases of SLE have been described during IFN treatment in immunocompetent patients treated for HCV infection[12]. In a series of 677 patients treated for HCV infection, 4% developed autoimmune diseases, the majority (62%) being represented by thyroid dysfunction; only 1 patient developed an SLE-like syndrome with an incidence of 0.15% in the study population[14]. Alfa-IFN therapy has been moreover associated with the development of idiopathic thrombocytopenic purpura, myasthenia gravis, Addison's disease, diabetes, COELIAC disease, rheumatoid arthritis, primary biliary cirrhosis, polymiositis, psoriasis[12,15-23].

Antiviral actions of alfa-IFN include the induction of several proteins, such as protein kinase (PKR) and 2',5' -oligoadenylate synthetase, important in viral dynamics[24]. Moreover, IFNs induce the expression of MHC both on antigen presenting cells (APC) and hepatocytes, resulting in a virus-specific lysis of infected cells mediated by cytotoxic T-cell response[25]. Thus, IFNs may lead to the development of autoimmune diseases by the up-regulation of MHC in both transplanted and non transplanted patients.

Moreover, the interaction of IFN activities in a particular pathway, such as in the immunosuppressed host, may lead to severe autoimmune manifestations that can compromise the graft survival. A relation between virological response and ACR was suggested in a recent study. Authors supposed that viral eradication improves microsomial function leading to a decrease of IS drugs levels[4]. The same mechanism may play a role in the induction of other autoimmune diseases such as that occurred to our patient who developed an SLE. Furthermore, our patient was on treatment with calcineurin inhibitors that have been thought to predispose to autoimmunity by interfering with T cell maturation and developing autoaggressive T-cells clones[26]. It is therefore possible that various mechanisms contribute to the development of autoimmune manifestations after liver transplantation.

In conclusion, the clinician should be aware of the possible development of autoimmune disorders, different from ACR, during interferon based treatment in LT patients, especially if signs of autoimmunity are present before starting IFN.

REFERENCES

1 **Clifford BD**, Donahue D, Smith L, Cable E, Luttig B, Manns M, Bonkovsky HL. High prevalence of serological markers of autoimmunity in patients with chronic hepatitis C. *Hepatology* 1995; **21**: 613-619

2 **Kerkar N**, Hadzić N, Davies ET, Portmann B, Donaldson PT, Rela M, Heaton ND, Vergani D, Mieli-Vergani G. De-novo autoimmune hepatitis after liver transplantation. *Lancet* 1998; **351**: 409-413

3 **Conti F**, Dousset B, Levillayer H, Gruska I, Weill B, Calmus Y. Autoantibodies after liver transplantation: a marker of allograft disease. *J Hepatol* 1997; **26**: 150A

4 **Kugelmas M**, Osgood MJ, Trotter JF, Bak T, Wachs M, Forman L, Kam I, Everson GT. Hepatitis C virus therapy, hepatocyte drug metabolism, and risk for acute cellular rejection. *Liver Transpl* 2003; **9**: 1159-1165

5 **Féray C**, Samuel D, Gigou M, Paradis V, David MF, Lemonnier C, Reynès M, Bismuth H. An open trial of interferon alfa recombinant for hepatitis C after liver transplantation: antiviral effects and risk of rejection. *Hepatology* 1995; **22**: 1084-1089

6 **Tan EM,** Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271-1277

7 **Hochberg MC**. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; **40**: 1725

8 Banff schema for grading liver allograft rejection: an international consensus document. *Hepatology* 1997; **25**: 658-663

9 **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Büschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938

10 **Fattovich G**, Giustina G, Favarato S, Ruol A. A survey of adverse events in 11,241 patients with chronic viral hepatitis treated with alfa interferon. *J Hepatol* 1996; **24**: 38-47

11 **Durelli L**, Ferrero B, Oggero A, Verdun E, Ghezzi A, Montanari E, Zaffaroni M. Thyroid function and autoimmunity during interferon beta-1b treatment: a multicenter prospective study. *J Clin Endocrinol Metab* 2001; **86**: 3525-3532

12 **Wilson LE**, Widman D, Dikman SH, Gorevic PD. Autoimmune disease complicating antiviral therapy for hepatitis C virus infection. *Semin Arthritis Rheum* 2002; **32**: 163-173

13 **Fabbri C**, Jaboli MF, Giovanelli S, Azzaroli F, Pezzoli A, Accogli E, Liva S, Nigro G, Miracolo A, Festi D, Colecchia A, Montagnani M, Roda E, Mazzella G. Gastric autoimmune disorders in patients with chronic hepatitis C before, during and after interferon-alpha therapy. *World J Gastroenterol* 2003; **9:** 1487-1490

14 **Okanoue T**, Sakamoto S, Itoh Y, Minami M, Yasui K, Sakamoto M, Nishioji K, Katagishi T, Nakagawa Y, Tada H, Sawa Y, Mizuno M, Kagawa K, Kashima K. Side effects of high-dose interferon therapy for chronic hepatitis C. *J Hepatol* 1996; **25**: 283-291

15 **Aspinall RJ**, Pockros PJ. The management of side-effects during therapy for hepatitis C. *Aliment Pharmacol Ther* 2004; **20**: 917-929

16 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

17 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982

18 **Hadziyannis SJ**, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355

19 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492

20 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499

21 **McHutchison JG**, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061-1069

22 **Fried MW**. Side effects of therapy of hepatitis C and their management. *Hepatology* 2002; **36**: S237-S244

23 **Dieperink E**, Willenbring M, Ho SB. Neuropsychiatric symptoms associated with hepatitis C and interferon alpha: A review. *Am J Psychiatry* 2000; **157**: 867-876

24 **Zeuzem S**, Schmidt JM, Lee JH, Rüster B, Roth WK. Effect of interferon alfa on the dynamics of hepatitis C virus turnover in vivo. *Hepatology* 1996; **23**: 366-371

25 **Samuel CE**. Antiviral actions of interferons. *Clin Microbiol Rev* 2001; **14**: 778-809, table of contents

26 **Damoiseaux JG**, van Breda Vriesman PJ. Cyclosporin A-induced autoimmunity: the result of defective de novo T-cell development. *Folia Biol* (Praha) 1998; **44**: 1-9

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CASE REPORT

A case of splenic abscess after radiofrequency ablation

Dimitris Zacharoulis, Emmanuel Katsogridakis, Constantinos Hatzitheofilou

Dimitris Zacharoulis, Emmanuel Katsogridakis, Constantinos Hatzitheofilou, Department of Surgery, University Hospital of Larissa, University of Thessaly, Greece
Correspondence to: Dimitris Zacharoulis, MD, University of Thessaly, University Hospital of Larissa, Mezourlo, Larissa 41222 Greece. zachadim@yahoo.com
Telephone: +30-6974-707500 Fax: +30-2410-670100
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Abstract

Radiofrequency ablation (RFA) is an innovative technique used primarily for the palliative treatment of unresectable liver tumors. Its therapeutic indications however, have been expanded and now include various other organs and diseases. There is a paucity of data regarding technical details and complications of the use of RFA in the spleen. We report a case of partial splenectomy using radiofrequency ablation for splenic hydatid disease, complicated by an abscess formation.

Key words: Radio frequency ablation; Partial splenectomy; Abscess; Hydatid disease

Zacharoulis D, Katsogridakis E, Hatzitheofilou C. A case of splenic abscess after radiofrequency ablation. *World J Gastroenterol* 2006; 12(26): 4256-4258

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INTRODUCTION

The spleen is an important component of the body's defenses against infection and the adverse consequences of its removal have become increasingly apparent over the last four to five decades. Death rates from overwhelming post splenectomy sepsis have been reported to be up to 600 times greater than those of the general population, with an estimated life time risk for post splenectomy sepsis of 5%. There is evidence that splenic reticuloendothelial function can be preserved by partial splenectomy, provided that at least 25% of splenic tissue is preserved[1,2].

Partial splenectomy is not routinely practiced mainly because of the lack of vascular control to arrest bleeding[3]. The most common indications included splenic trauma, staging and palliation of malignant diseases, inflammatory pseudotumors, hemangiomas, lipomas, hamartomas, parasitic and non parasitic cysts and other benign conditions.

Radiofrequency ablation is a relatively new technique for local ablation of unresectable liver tumors[4]. It has been successfully adapted as a treatment modality of primary and metastatic tumors of the liver, kidney, breast, and bone[5]. Furthermore, the RF energy has been used successfully for the division of liver parenchyma[6]. Very few clinical studies refer to the use of RF energy in the spleen. We report a case of partial splenectomy using RF energy complicated by an abscess formation.

CASE REPORT

A 52 year old man was admitted to our hospital with vague abdominal pain localized to the left upper quadrant of the abdomen. The patient's past medical history included an appendectomy performed 30 years ago and two operations for the removal of liver echinococcal cysts, performed 28 and 4 years ago. Physical examination and routine blood tests were unremarkable. The patient was found to be positive for anti-echinococcal antibodies, and thus two cycles of scolicidal agent were administered pre-operatively. The patient's radiographic work up, which included routine X-rays, an ultrasound and a computed tomography of the abdomen, revealed two hydatid cysts; one located at the lower splenic pole (9 cm × 10 cm × 8 cm) and the other located at the left pelvic floor (10 cm × 5 cm × 4 cm).

The patient underwent an exploratory laparotomy, through a midline incision. The abdominal cavity was entered with difficulty due to dense adhesions. The left colon was mobilized from the lateral abdominal wall, but the cyst was attached to the mesentery and subsequently its blood supply was sacrificed. Multiple erosions of the left colon were identified and subsequently a left colectomy and end colostomy were performed. The cyst was finally removed en block.

The spleen was surrounded by multiple dense adhesions and access to the splenic hilum was very difficult. However all the surrounding tissue was mobilized and pushed away from the cyst and the splenic pole. No vascular division took place. The cyst involved the lower pole parenchyma.

At this point division of the splenic parenchyma was decided in order to achieve en block removal of the hytadid cyst.

The Radionics Cooltip Radiofrequency System (Radionics/Tyco Hellas) was used to create a zone of desiccation between the cyst and the splenic

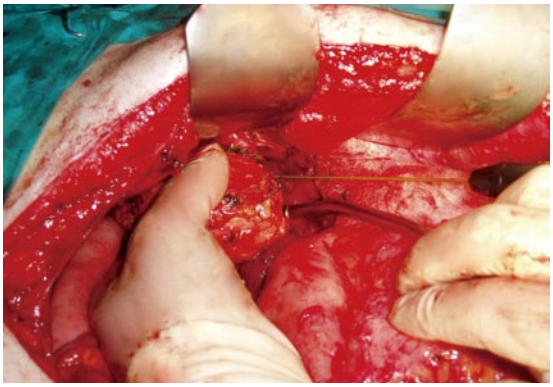

Figure 1 A zone of dessication was created between the hytadid cyst and the splenic parenchyma using the RF electrode.

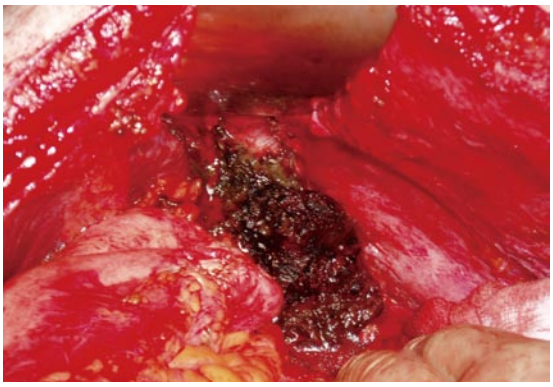

Figure 2 The splenic remnant after removal of the hytadid cyst and hemostasis using the radiofrequency energy.

parenchyma. A 3 cm Cooltip RF electrode was applied approximately 2 cm from the cyst wall and into the splenic parenchyma away from the hilum (Figure 1). The RF generator was turned on to the maximum power up to 140 W. Generator output, tissue impedance and electrode tip temperature were calculated, and measurement appeared on the generator's screen. Chilled saline was circulated by the perfusion pump through two coaxial cannulae in the probe during RF application to help prevent tissue boiling and cavitations immediately adjacent to the needle. The generator was activated to the maximum power and using the manual mode, the RF was applied until the impedance of the coagulated tissue reached a level twice as high as the initial measurement. Division of the splenic parenchyma was carried out using scissors. Residual bleeding was taken care of using RF again. The cyst was finally removed en block (Figure 2).

After copious irrigation and hemostasis the abdominal cavity was drained and closed in a standard fashion and the colostomy was matured. The pathology revealed macroscopically an intact hydatid cyst surrounded by approximately a 1 cm ring of coagulated splenic tissue.

For the first two post operative days, small amounts of dark bloody stained fluid was continuously flowing from the splenic bed drain. On the fourth postoperative day the patient became septic and he was transferred to the ICU in septic shock.

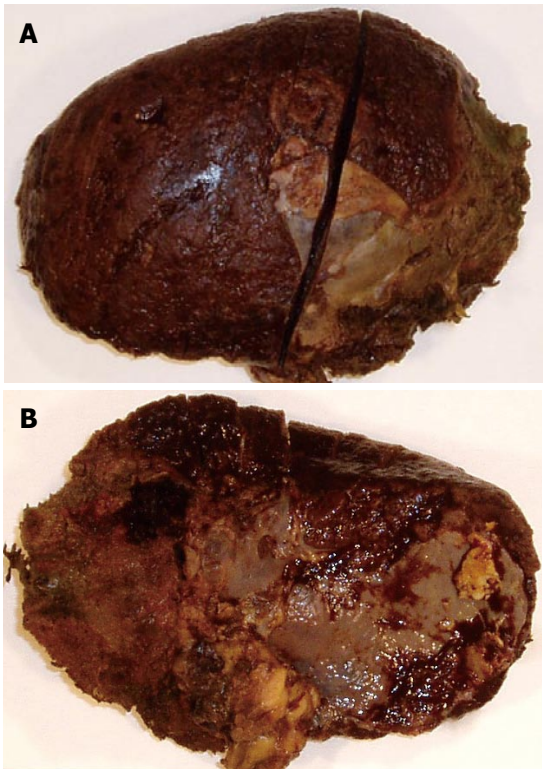

Figure 3 The splenic remnant was found to be infected with multiple microabscesses.

Urgent abdominal CT revealed the presence of atelectasis of the lower lobe of the left lung, with pleural effusion, gastric dilation and the presence of perisplenic abscess.

The patient was taken back to the operating room, and splenectomy was performed. The spleen was found to be infected and multiple abscesses surrounding the spleen were identified (Figure 3). The abdominal cavity was then washed out with copious amount of irrigation, and drains were placed again.

After 3 d in the Intensive Care Unit, the patient returned to the floor. Subsequently, the patient developed an enterocutaneous fistula, which was treated conservatively.

The pathology macroscopically revealed a splenic remnant that weighed 305 g and was 15 cm × 12 cm × 3 cm in size. A section of 1 cm of the parenchyma showed ash colored areas throughout the spleen, which became more prominent towards the lower pole.

Microscopically a zone of coagulative necrosis with deeper areas of liquefaction and hemorrhagic necrosis was identified. The rest of the spleen was characterized by changes in the architecture of the white pulp. The red pulp was characterized by intense dilation of the sinuses with infiltration of plasma cells and macrophages around the perivascular space throughout the spleen.

DISCUSSION

The incidence of septic complications, such as abscess formation, in RF assisted operations has been reported in various organs. In a recent review of 3670 patients

with liver tumors who underwent open, laparoscopic percutaneous or combined RFA the incidence of septic complications was 1.1%[7]. Although there are no large series of RF assisted hepatectomies the reported incidence of an abscess formation after division of the liver parenchyma using RF energy remains low.

Septic complications resulting from the application of RF energy to the kidney, adrenals, breast and lungs have been documented to have an incidence of 1%. However all the reported studies have been conducted on a small series of patients[5].

Predisposing factors for septic complications in RF assisted operations are diabetes mellitus, the existence of bilio-enteric anastomosis for the liver cases and a simultaneous "dirty" operation, like a colectomy, with the most common reported pathogen being the *Staphylococcus aureus*[8].

In the presented case a colectomy was performed with a subsequent end colostomy due to multiple adhesions transforming a clean procedure into a "dirty one". In our opinion, that may be a predisposing factor for a septic complication following RF assisted partial splenectomy, though this has not been investigated in the literature in most series, since most of the studies refer to RF as a single procedure.

Intraoperative cultures of the abscess material obtained during the operation, grew an enteric flora *Enterococcus Faecium,* sensitive to a broad spectrum of antibiotics.

Experimental studies[9,10] have established the safety and efficiency of RF energy to splenic tissue. Quanda Liu *et al*[11] reported the first clinical series of nine patients who underwent RFA for hypersplenism in patients with liver cirrhosis, with no mortality and no morbidity, other than hydrothorax. However, in this study, the spleen was ablated but not divided. To the best of our knowledge, no data exists regarding the application of the RF energy for partial splenectomy to the splenic parenchyma other than few case reports with zero reported morbidity[3,12].

We report a case of partial splenectomy using RF energy for hydatid cyst removal complicated by a splenic abscess.

Based on our experience of RF assisted liver resections, we applied maximum RF energy to create a zone of desiccation on the splenic parenchyma. However, the application of RF energy to the spleen is in many ways different than that performed on the liver parenchyma. Perhaps the double blood supply to the liver and subsequently the hit sink phenomenon, makes the liver parenchyma less sensitive to the RF energy than that of the spleen. In our case the pathology of the splenic remnant revealed changes throughout the splenic parenchyma probably due to excessive applied energy.

In conclusion, we believe that RF assisted partial splenectomy should be performed with the least possible RF energy to achieve hemostasis.

REFERENCES

1 **Malangoni MA**, Dawes LG, Droege EA, Rao SA, Collier BD, Almagro UA. Splenic phagocytic function after partial splenectomy and splenic autotransplantation. *Arch Surg* 1985; **120**: 275-278

2 **Traub A**, Giebink GS, Smith C, Kuni CC, Brekke ML, Edlund D, Perry JF. Splenic reticuloendothelial function after splenectomy, spleen repair, and spleen autotransplantation. *N Engl J Med* 1987; **317:** 1559-1564

3 **Habib NA**, Spalding D, Navarra G, Nicholls J. How we do a bloodless partial splenectomy. *Am J Surg 2003;* **186**: 164-166

4 **Liu QD**, Ma KS, He ZP, Ding J, Huang XQ, Dong JH. Experimental study on the feasibility and safety of radiofrequency ablation for secondary splenomagely and hypersplenism. *World J Gastroenterol* 2003; **9**: 813-817

5 **Rhim H**, Dodd GD 3rd, Chintapalli KN, Wood BJ, Dupuy DE, Hvizda JL, Sewell PE, Goldberg SN. Radiofrequency thermal ablation of abdominal tumors: lessons learned from complications. *Radiographics* 2004; **24**: 41-52

6 **Zacharoulis D**, Asopa V, Navarra G, Nicholls JP, Jensen SL, Habib NA. Hepatectomy using intraoperative ultrasound-guided radiofrequency ablation. *Int Surg* 2003; **88**: 80-82

7 **Decadt B**, Siriwardena AK. Radiofrequency ablation of liver tumours: systematic review. *Lancet Oncol* 2004; **5**: 550-560

8 **Lencioni R**, Crocetti L, Cioni D, Della Pina C, Bartolozzi C. Percutaneous radiofrequency ablation of hepatic colorectal metastases: technique, indications, results, and new promises. *Invest Radiol* 2004; **39**: 689-697

9 **Felekouras E**, Kontos M, Pissanou T, Pikoulis E, Drakos E, Papalambros E, Diamantis T, Bastounis E. A new spleen-preserving technique using radiofrequency ablation technology. *J Trauma* 2004; **57**: 1225-1229

10 **Haghighi KS**, Steinke K, Hazratwala K, Kam PC, Daniel S, Morris DL. Controlled study of in-line ovine spleen transection assisted by radiofrequency ablation. *J Trauma* 2005; **58**: 841-844

11 **Liu Q**, Ma K, He Z, Dong J, Hua X, Huang X, Qiao L. Radiofrequency ablation for hypersplenism in patients with liver cirrhosis: a pilot study. *J Gastrointest Surg* 2005; **9**: 648-657

12 **Velanovich V,** Weaver M. Partial splenectomy using a coupled saline-radiofrequency hemostatic device. *Am J Surg* 2003; **185**: 66-68

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PO Box 2345, Beijing 100023, China
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CASE REPORT

Primary melanoma of the gallbladder: Does it exist? Report of a case and review of the literature

Michail Safioleas, Emmanouil Agapitos, Konstantinos Kontzoglou, Michail Stamatakos, Panagiotis Safioleas, George Mouzopoulos, Alkiviadis Kostakis

Michail Safioleas, Konstantinos Kontzoglou, Michail Stamatakos, Panagiotis Safioleas, Alkiviadis Kostakis, 2nd Department of Propedeutic Surgery, School of Medicine, Athens University, Laiko Hospital, Greece
Emmanouil Agapitos, Depatment of Pathology, School of Medicine, Athens University, Greece
George Mouzopoulos, Department of General Surgery, General Hospital of Sparta, Greece
Correspondence to: Professor Michail Safioleas, MD, PhD, 7 Kyprou Ave. Filothei, 15237, Athens, Greece. stamatakosmih@yahoo.gr
Telephone: +30-210-6812188
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Abstract

With the occasion of a case of malignant melanoma of the gallbladder, which appeared to be primary, we have reviewed the literature and the result of this research was that primary melanoma of the gallbladder remains a questionable medical entity. Only few cases of both primary and metastatic gallbladder melanoma have been reported so far, and the only agreement is that surgery is the mainstay treatment. The role of adjuvant chemotherapy, hormonotherapy or immunotherapy for both primary and metastatic disease remains undefined.

Key words: Neoplasms of the gallbladder; Gallbladder; Metastatic melanoma

Safioleas M, Agapitos E, Kontzoglou K, Stamatakos M, Safioleas P, Mouzopoulos G, Kostakis A. Primary melanoma of the gallbladder: Does it exist? Report of a case and review of the literature. *World J Gastroenterol* 2006; 12(26): 4259-4261

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INTRODUCTION

Having a biopsy specimen histologically proving melanoma of the gallbladder, it is difficult to determine whether it is a primary or secondary tumor. Even though gallbladder and this metastatic lesion comprises between 50%-66% of gallbladder metastasis[1], the occurrence of metastasis in the gallbladder is rare and has only been reported in the literature exceptionally[2-7]. Both primary melanoma in the gallbladder and isolated metastasis to the gallbladder, are not common events. In fact, primary melanoma in the gallbladder is an extremely rare condition and its existence is still questioned by some. According to the literature, primary malignant melanoma in the gallbladder is a controversial issue, and it is still far from clear whether primary melanoma of this organ is a definite entity in itself. Herein, we present a patient in whom malignant melanoma developed initially in the gallbladder. There was no evidence of another acceptable primary site in postoperative investigation and no previous history could be obtained of a pigment cutaneous lesion, which had been treated or regressed.

CASE REPORT

A 38-year old woman presented with a 36-h history of right upper abdominal pain associated with nausea, vomiting and a temperature 38.6℃. Physical examination revealed the presence of severe tenderness in the right upper quadrant of the abdomen. The gallbladder was not palpable. The hematological tests showed an elevation of her white blood cell count (WBC) to 13 800/mm^3 with neutrophilia. Liver function studies were normal; these laboratory findings disclosed the following values (the normal reference range is given in parentheses): serum aspirate aminotransferase, 21 IU/L (< 30 IU/L); alanine aminotransferase, 19 IU/L (< 30 IU/L); serum alkaline phosphatase, 184 IU/L (100-320 IU/L); serum g-glutamyl transpeptidase, 30 IU/L (16-73 IU/L); and the serum bilirubin, 0.7 mg/dL (< 1 mg/dL). Ultrasound examination of the gallbladder did not demonstrate lithiasis but a polypoid mass without acoustic shadowing (Figure 1). The patient underwent open cholecystectomy. The specimen was opened and a polypoid mass measuring 1.5 cm × 2.0 cm of dark-yellow color was revealed. The postoperative course of the patient was uneventful, but the histological report indicated a melanoma of the gallbladder (Figures 2 and 3). In H&E stain the diagnosis of a primary melanoma was suspicious, confirmed by immunostaining (HMB-45). After that, a meticulous medical history, physical examination and imaging check did not reveal any other primary focus of the disease. No evidence of the disease was marked after six months in the periodical control, however, four months later the patient died from cerebral hemorrhage due to brain metastasis. Although the patient was informed of her disease, she refused any chemotherapy as well as

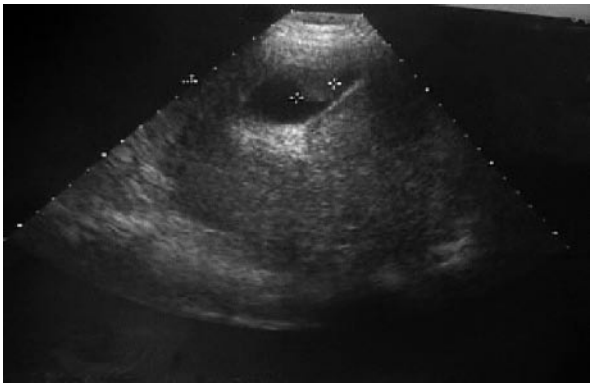

Figure 1 Ultrasound of melanoma of gallbladder.

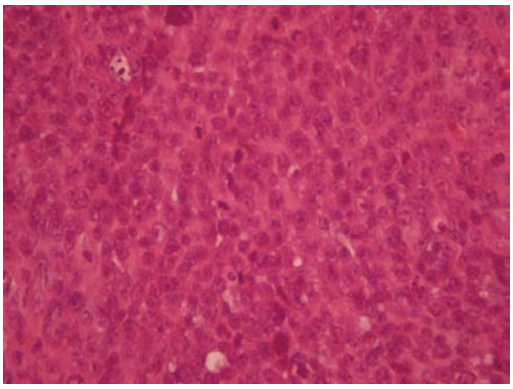

Figure 2 Histological appearance of melanoma of gallbladder stained with HE. × 400.

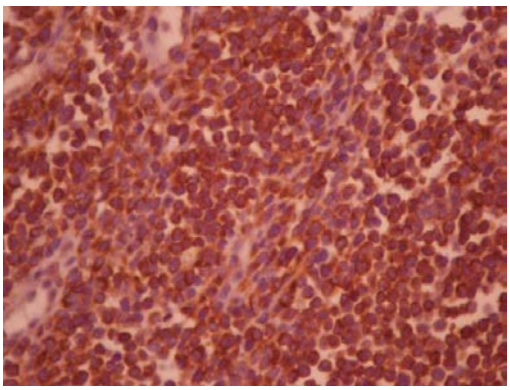

Figure 3 Neoplastic cells immunostained with anti-HMB-45 antibodies. × 400.

the proposed transplenic immunostimulation[8,9].

DISCUSSION

Theoretically, according to histogenesis, melanoblasts have not yet been demonstrated in organs of endodermal origin[10] and as gallbladder is an endodermal derivative, it is unlikely that melanoma arises from this organ or other visceral structures. On the other hand, non neoplastic melanoblasts resulting from migration of melanin-producing cells from the neural crest to endodermal derivatives during embryologic development explains the presence of melanocytes within their mucosa[11-13] and supports the possibility of developing primary melanomas at these sites[14]. Therefore, primary melanoma of the gallbladder is at least theoretically possible[4,15]. Even though primary melanoma of the gallbladder remains a debatable clinical entity, reports on this subject continue to accrue. In 1907 the first primary melanoma of the gallbladder was described by *Wieting* and *Handt*[16] and fifty years later *Walsh* reported the first histologically proven case of primary gallbladder melanoma[17]. Since that time there have been additional reports of primary melanoma of the gallbladder[12,15,18-20]. In fact, it is very difficult to differentiate primary from metastatic lesions based on histological features. *Mac Fadden et al*, compared primary and metastatic biliary melanomas and found that the two groups displayed remarkable similarity with regard to pathologic findings[13]. To help in the differentiation between primary and metastatic melanoma of the gallbladder, according to the literature, the following clinical and pathological criteria should be fulfilled: (1) any other obvious primary site must be excluded by medical history and laboratory investigation, and (2) the tumor, either papillary or polypoid, must be solitary arising from the mucosa of the gallbladder and display junctional activity[2-4]. Junctional activity, i.e, intraepithelial extension in the mucosa overlying the tumor is considered as significant finding in the diagnosis of the primary melanoma of mucosal surfaces[11,21]. However, the presence of atypical melanocytes in the adjacent epithelium of invasive tumor does not necessarily define a primary tumor[22]. Although the presence of "junctional changes" of the mucosa is still controversial, the presence of a solitary polypoid tumor within the lumen of the gallbladder accompanied by junctional activity within the tumor and the absence of an antecedent history of melanoma, or lack of another obvious primary site, are factors that suggest the gallbladder as the primary site of origin. Finally, even today, the establishment of primary histogenesis in these cases is contingent upon a detailed medical history of the patient, a thorough cutaneous and ophthalmic inspection and a radiologic control. However, there always exists the possibility that a regressed primary tumor is a hidden source of gallbladder melanoma. In regard to clinical presentation, involvement of the gallbladder rarely produces symptoms, which could explain the paucity of cases reported in the literature[23], however, as it is usually reported including our case, acute cholecystitis without the presence of stone in the majority of cases was the initial clinical manifestation and the reason for emergency admission in the hospital. In contrast to gallbladder cancer, melanoma of the gallbladder does not seem to be associated to cholelithiasis. In 1999 *Dong et al*, after having reviewed the literature, found that gallstones appear to be only incidentally and namely in 21.1% of cases (4 of 19 patients) with primary lesions and 27.3% of cases (3 of 11 patients) with symptomatic metastatic disease and this was consistent with the results of their series[24]. Several diagnostic modalities have been employed to investigate gallbladder lesions. Ultrasound is by far the most useful tool in this attempt. Upper ultrasound abdominal examinations are frequently requested in patients with thick high grade malignant melanoma or clinical suspicion of metastasis. Ultrasound may document metastatic lesions

within the gallbladder. However, polypoid lesions in the lumen of the gallbladder present a focal thickening of the gallbladder wall without acoustic shadowing, due to their lower density in relation with gallstones. As autopsy studies have confirmed the incidence of the gallbladder metastasis from malignant melanoma to be 15%, a careful review of the gallbladder is advocated when abdominal ultrasound examination is performed on patient with malignant melanoma. Ultrasound helps to distinguish between metastasis and benign polyps. The importance of size of the lesion is obvious as 94% of the benign lesions are less than 1 cm in diameter, whereas, 88% of malignant lesion are more than 1 cm[25]. If the presence of vascular flow within the mass itself is documented, the usefulness of colour-flow Doppler ultrasonography is evident[26]. Computed tomography is of some value especially in detecting metastatic disease.

Surgical excision is the treatment of choice of both primary and metastatic melanomas of gallbladder. During surgery a thorough search is needed in order to detect any possible abdominal metastasis, as the majority of gallbladder metastases are totally asymptomatic. Although in most cases these metastases are in the form of multiple serosal implants, there are intraluminal metastases involving the mucosa and this fact creates difficulties in detecting these metastases during surgery. Although the mainstay of treatment is surgical removal of the gallbladder, we believe that study of the clear role of adjuvant chemotherapy, hormonotherapy or immunotherapy at increasing the survival rate is necessary. Basically, the presence of multiple metastases is an indication of systematic chemotherapy. Till today, the usefulness of adjuvant chemotherapy for both primary and metastatic melanomas remains to be clarified, as only one controlled trial has shown significant improvement in response rate mid survival with the combination dacarbazine and tamoxifen[27]. Despite appropriate therapy, the diagnosis of either condition portends a poor prognosis with few patients surviving more than two years. Furthermore, according to *Langley*, the prognosis of metastatic melanoma is dismal and the average survival is 7 mo[28].

In conclusion, firstly, the outlook of patients with gallbladder melanoma remains bleak and secondly, the existence of primary melanoma of the gallbladder is still questionable, and certainly will not be settled by this article.

REFERENCES

1 **Backman H**. Metastases of malignant melanoma in the gastrointestinal tract. *Geriatrics* 1969; **24**: 112-120
2 **Swiatoniowski G**, Mazur G, Hałoń A, Rozumek G, Dabrowska M, Zawisza R, Prudlak E. Malignant melanoma with gall bladder metastasis as a second neoplasm in the course of prostate cancer. *Pathol Oncol Res* 2004; **10**: 243-245
3 **Heath DI**, Womack C. Primary malignant melanoma of the gall bladder. *J Clin Pathol* 1988; **41**: 1073-1077
4 **Higgins CM**, Strutton GM. Malignant melanoma of the gall bladder--does primary melanoma exist? *Pathology* 1995; **27**: 312-314
5 **Köhler U**, Jacobi T, Sebastian G, Nagel M. [Laparoscopic cholecystectomy in isolated gallbladder metastasis of malignant melanoma]. *Chirurg* 2000; **71**: 1517-1520
6 **Ostick DG**, Haqqani MT. Obstructive cholecystitis due to metastatic melanoma. *Postgrad Med J* 1976; **52**: 710-712
7 **Seelig MH**, Schönleben K. Laparoscopic cholecystectomy for a metastasis of a malignant melanoma in the gallbladder. *Z Gastroenterol* 1997; **35**: 673-675
8 **Boura P**, Kountouras J, Lygidakis NJ, Safioleas M, Aphinives P. Transplenic and transtumoral in vivo immunostimulation: effect on cellular immunity parameters. *Hepatogastroenterology* 1999; **46**: 799-803
9 **Lygidakis NJ**, Safioleas M, Sgourakis G, Spentzouris N, Dedemadi G, Sotiropoulou P. Metastatic melanoma: transplenic immunostimulation--a new therapeutical alternative for an ever-challenging disease. *Hepatogastroenterology* 1999; **46**: 148-156
10 **Willis RA**. Spread of tumor in the human body. 2nd Ed. London: Butterworth, 1952
11 **McFadden PM**, Krementz ET, McKinnon WM, Pararo LL, Ryan RF. Metastatic melanoma of the gallbladder. *Cancer* 1979; **44**: 1802-1808
12 **Carle G**, Lessells AM, Best PV. Malignant melanoma of the gallbladder: a case report. *Cancer* 1981; **48**: 2318-2322
13 **Naguib SE**, Aterman K. Presumed primary malignant melanoma of the gallbladder. Report of a case and a review of literature. *Am J Dermatopathol* 1984; **6** Suppl: 231-243
14 **DASGUPTA T**, BRASFIELD R. METASTATIC MELANOMA. A CLINICOPATHOLOGICAL STUDY. *Cancer* 1964; **17**: 1323-1339
15 **Peison B**, Rabin L. Malignant melanoma of the gallbladder: report of three cases and review of the literature. *Cancer* 1976; **37**: 2448-2454
16 **Wieting and Hamdi**. Uber die physiologische and pathologische melanipigentierund und den epithelialen ursprung der melanoblastome: Ein primares melanoblastom der gallenblase. *Beitr Path Anat* 1907; **42**: 23-84
17 **WALSH TS Jr**. Primary melanoma of the gallbladder with cervical metastasis and fourteen and a half year survival; first histologically proved case. *Cancer* 1956; **9**: 518-522
18 **Sierra-Callejas JL**, Warecka K. Primary malignant melanoma of the gallbladder. *Virchows Arch A Pathol Anat Histol* 1976; **370**: 233-238
19 **Hayashi K**. [PCR-SSCP--rapid and easy detection of DNA-sequence changes]. *Hum Cell* 1992; **5**: 180-184
20 **Borja SR**, Meyer WR, Cahill JP. Malignant melanoma of the gallbladder. Report of a case. *Cancer* 1984; **54**: 929-931
21 **ALLEN AC**, SPITZ S. Malignant melanoma; a clinicopathological analysis of the criteria for diagnosis and prognosis. *Cancer* 1953; **6**: 1-45
22 **Murphy MN**, Lorimer SM, Glennon PE. Metastatic melanoma of the gallbladder: a case report and review of the literature. *J Surg Oncol* 1987; **34**: 68-72
23 **Velez AF**, Penetrante RB, Spellman JE Jr, Orozco A, Karakousis CP. Malignant melanoma of the gallbladder: report of a case and review of the literature. *Am Surg* 1995; **61**: 1095-1098
24 **Dong XD**, DeMatos P, Prieto VG, Seigler HF. Melanoma of the gallbladder: a review of cases seen at Duke University Medical Center. *Cancer* 1999; **85**: 32-39
25 **Holloway BJ**, King DM. Ultrasound diagnosis of metastatic melanoma of the gallbladder. *Br J Radiol* 1997; **70**: 1122-1125
26 **Avila NA**, Shawker TH, Fraker D. Color-flow Doppler ultrasonography in metastatic melanoma of the gallbladder. *J Clin Ultrasound* 1994; **22**: 342-347
27 **Rusthoven JJ**. The evidence for tamoxifen and chemotherapy as treatment for metastatic melanoma. *Eur J Cancer* 1998; **34** Suppl 3: S31-36
28 **Langley RG**, Bailey EM, Sober AJ. Acute cholecystitis from metastatic melanoma to the gall-bladder in a patient with a low-risk melanoma. *Br J Dermatol* 1997; **136**: 279-282

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PO Box 2345, Beijing 100023, China
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CASE REPORT

Multiple small bowel ruptures due to ischemic enteritis: A case report

Stylianos Delikoukos, Gregorios Christodoulidis, Dimitrios Zacharoulis, Antigoni Poultsidi, Constantine Hatzitheofilou

Stylianos Delikoukos, Gregorios Christodoulidis, Dimitrios Zacharoulis, Antigoni Poultsidi, Constantine Hatzitheofilou, Department of Surgery, Larissa University Hospital, Larissa, Greece
Correspondence to: Stylianos Delikoukos, MD, PhD, 9 Papakiriazi Str, Larissa 41 223, Greece. morfula@otenet.gr
Telephone: +30-2410-625636 Fax: +30-2410-670100
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Abstract

A rare case of multiple small bowel ruptures due to ischemic enteritis (ISE) is reported. The patient was admitted to the hospital with acute abdominal pain followed by bloody diarrhoeas. Preoperative colonoscopic findings were similar to those presented in Crohn's disease. Intraoperatively, ischemic lesions and multiple ruptures were localized at the jejunum and the proximal ileum. Histopathological examination of the resected bowel segment established the diagnosis of ISE. Although ISE is not common, concurred multiple ruptures of the small bowel is a rare but actual complication.

Key words: Multiple small bowel ruptures; Ischemic enteritis

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INTRODUCTION

Ischemic enteritis (ISE) is caused either by interruption or significant decrease of the arterial inflow to the small intestine. Most patients are elderly, i.e., over sixty years of age. Younger patients, however, especially those with diabetes, lupus erythematosus or sickle-cell anaemia, may also present with ISE. Definite diagnosis of ISE cannot be established before histopathological results are obtained. ISE seems to be a rapid, progressive disease resulting in a 95% mortality rate[1]. We report a rare case of acute abdomen due to multiple ruptures of the small bowel. ISE was proved to be the cause of the ruptures.

CASE REPORT

A 62-year old male presented with a two-month history of abdominal pain, watery diarrhoeas and occasionally fresh intestinal bleeding. Colonoscopy revealed aphthous ulcers both in the ascending colon and in the cecum. A small bowel series showed "tile-paved" images with multiple ulcers in the ileum. Furthermore, gastroscopy revealed the presence of aphthous ulcers in both the stomach and the duodenum. These ulcers were similar to those found in the colon. Biopsy specimens were taken from the stomach, the ileum, the cecum and the rest of the colon. The diagnosis of Crohn's disease was suspected and anti-Crohn's drug therapy was started. The histopathology results 8 d later, however, excluded Crohn's disease and revealed signs of focal enteritis without inflammatory reaction in the submucosa. Ten days after the first admission, the patient presented again in the emergency department suffering from acute abdominal pain accompanied by diarrhoeas and moderate intestinal bleeding. Physical examination revealed acute abdomen and the plain abdominal X-rays showed free air under the diaphragm. CT scanning confirmed liquid and air accumulation in the abdominal cavity.

The patient underwent emergency laparotomy. Intraoperatively, more than twenty micro-perforations localized in the mesenteric surface of the small bowel were found extending from the end of the jejunum to the middle of the ileum. Intestinal resection (about 60 cm in length) followed by side-to-side anastomosis was performed. Four days later, the patient developed similar symptoms of acute abdomen. On reoperation, numerous micro-perforations in both the jejunum and the remaining ileum were found. An additional small bowel resection (about 80 cm in length) was performed, followed by jejunostomy.

On the third postoperative day the patient was operated on again due to the same symptoms. Multiple perforations were encountered once more in the remaining small bowel and an additional bowel resection (approximately 120 cm in length) was performed. Within three days the patient developed renal and hepatic failure and died 2 d later. Macroscopically, the intestinal lesions were characterized by mucosal ulceration of variable length along the resected segments of small bowel, progressing to full thickness necrosis of the intestinal wall. Histopathological examina-

tion revealed numerous scattered segments of acute necrotizing enteritis with annular strictures and relatively shallow ulcers.

DISCUSSION

Two types of ISE have been described in the literature: occlusive and non-occlusive. Embolism or thrombosis of the superior mesenteric artery are the causes of occlusion in 30% and 25% of the patients respectively. Thrombosis of the superior mesenteric vein is responsible for occlusion in 20% of the patients. Non-occlusive ISE occurs in the remaining patients (25%)[2]. Although the embolus in superior mesenteric artery embolism is usually installed in the middle colic artery, small intestinal branches are also sometimes occluded. The jejunum is affected in 20% and the ileum in 45%-55% of the patients respectively[3]. Thrombosis of the superior mesenteric artery or vein is considered to be idiopathic in the majority of patients. However, it can also be related to diseases such as portal hypertension, septicaemia, lack of antithrombin Ⅲ, lack of protein C or S, as well as chronic contraceptive pills intake[4].

Non-occlusive ISE is due to low blood flow because of cardiac insufficiency, acute myocardial infarction, arrhythmias, dissecting aneurysm of the aorta, septicaemia and diabetes mellitus as well. Decrease of blood flow up to 80% of its normal value occurs due to prolonged vasoconstriction of the mesenteric vessels[2]. According to Feurle *et al*[5], analogous to ischemic colitis, an entity of acute ischemic small bowel enteritis exists, so that mesenteric ischemia apparently can induce a clinical syndrome of "regional enteritis", as occurred in our patient.

Early symptoms (acute abdominal pain and bloody diarrhoeas) and late complications (intestinal necrosis, peritonitis, septicaemia and shock) in our patient, were similar to those presented in the literature[6].

The accuracy of angiography in localizing the site of embolism or thrombosis of the superior mesenteric artery is more than 95%. Its main advantage is that the catheter may remain in the artery, so that vasodilatation substances can be properly given[7]. Angiography was not performed in our case due to acute abdominal symptoms. These symptoms led the patient to emergency laparotomy. The accuracy of CT scans in diagnosing mesenteric artery or vein thrombosis is approximately 80%. For embolism the rate is 45%-55%[8,9]. CT scans, however, fail to establish the diagnosis in the majority of ISE cases, and this happened in our patient as well. ISE due to superior mesenteric artery embolism can be treated by arteriotomy and embolectomy. The patient should undergo an aorto-mesenteric bypass in the case of thrombosis of the superior mesenteric artery. In initial stages of superior mesenteric vein thrombosis conservative treatment with high doses of heparin is recommended. Unfortunately, in most cases the diagnosis is established during laparotomy, due to acute abdominal symptoms. This occurred in our patient as well[10].

In conclusion, ischemic enteritis is an uncommon but usually fatal clinical condition. The disease may lead to haemorrhage, intestinal necrosis, multiple small bowel ruptures, peritonitis, septicaemia, shock and death. Immediate diagnosis and proper medical and surgical therapy are crucial for a good prognosis. Unfortunately, a definite diagnosis of the disease is usually established after histopathological results of the resected bowel segment have been obtained. Although the disease is not common, it should be considered in the differential diagnosis of acute abdomen, especially when acute abdominal pain is accompanied by diarrhoea and/or intestinal bleeding.

REFERENCES

1 **Clavien PA**. Diagnosis and management of mesenteric infarction. *Br J Surg* 1990; **77**: 601-603
2 **Levy PJ**, Krausz MM, Manny J. Acute mesenteric ischemia: improved results--a retrospective analysis of ninety-two patients. *Surgery* 1990; **107**: 372-380
3 **Kitchens CS**. Evolution of our understanding of the pathophysiology of primary mesenteric venous thrombosis. *Am J Surg* 1992; **163**: 346-348
4 **Chen MC**, Brown MC, Willson RA, Nicholls S, Surawicz CM. Mesenteric vein thrombosis. Four cases and review of the literature. *Dig Dis* 1996; **14**: 382-389
5 **Feurle GE**, Haag B. Acute small bowel ischemia without transmural infarction. *Z Gastroenterol* 1991; **29**: 349-352
6 **Wig JD**, Malik AK, Suri S, Singh K, Talwar BL. Acute segmental enteritis. *J Diarrhoeal Dis* Res 1984; **2**: 37-40
7 **Chandrasekharam VV**, Srivastava DN, Mathur M, Bhatnagar V. Angiographic and immunologic studies in acute necrotizing jejunoileitis. *J Trop Pediatr* 2002; **48**: 88-92
8 **Shaff MI**, Tarr RW, Partain CL, James AE Jr. Computed tomography and magnetic resonance imaging of the acute abdomen. *Surg Clin North Am* 1988; **68**: 233-254
9 **Mallo RD**, Salem L, Lalani T, Flum DR. Computed tomography diagnosis of ischemia and complete obstruction in small bowel obstruction: a systematic review. *J Gastrointest Surg* 2005; **9**: 690-694
10 **Boley SJ**, Feinstein FR, Sammartano R, Brandt LJ, Sprayregen S. New concepts in the management of emboli of the superior mesenteric artery. *Surg Gynecol Obstet* 1981; **153**: 561-569

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PO Box 2345, Beijing 100023, China
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wjg@wjgnet.com

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CASE REPORT

Splenic arteriovenous fistula and sudden onset of portal hypertension as complications of a ruptured splenic artery aneurysm: Successful treatment with transcatheter arterial embolization. A case study and review of the literature

Dimitrios Siablis, Zafiria G Papathanassiou, Dimitrios Karnabatidis, Nikolaos Christeas, Konstantinos Katsanos, Constantine Vagianos

Dimitrios Siablis, Zafiria G Papathanassiou, Dimitrios Karnabatidis, Nikolaos Christeas, Konstantinos Katsanos, Constantine Vagianos, Departments of Interventional Radiology and Surgery, University Hospital of Patras, Rio, Greece
Correspondence to: Professor Dimitrios Siablis, MD, Department of Radiology, Medical School, University of Patras, Rio 26504 Patras, Greece. papaze@otenet.gr
Telephone: +30-261-450723 Fax: +30-261-993987
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Abstract

Splenic arteriovenous fistula (SAVF) accounts for an unusual but well-documented treatable cause of portal hypertension[1-4]. A case of a 50-year-old multiparous female who developed suddenly portal hypertension due to SAVF formation is presented. The patient suffered from repeated episodes of haematemesis and melaena during the past twelve days and thus was emergently admitted to hospital for management. Clinical and laboratory investigations established the diagnosis of portal hypertension in the absence of liver parenchymal disease. Endoscopy revealed multiple esophageal bleeding varices. Abdominal computed tomography (CT) and transfemoral celiac arteriography documented the presence of a tortuous and aneurysmatic splenic artery and premature filling of an enlarged splenic vein, findings highly suggestive of an SAVF. The aforementioned vascular abnormality was successfully treated with percutaneous transcatheter embolization. Neither recurrence nor other complications were observed.

Key words: Splenic artery aneurysm; Splenic arteriovenous fistula; Portal hypertension; Transcatheter embolization

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INTRODUCTION

Splenic arteriovenous fistulas (SAVF) represent a rare pathological entity that should be suspected especially in cases of acute portal hypertension not related to chronic liver disease[1-4]. They may be either asymptomatic or lead rapidly to the development of portal hypertension, with a clinical picture of gastro-intestinal bleeding, diarrhea, ascites or unfavorable heart failure due to hyperdynamic blood flow state[1-4].

In this report an uncommon case of portal hypertension affecting a multiparous female is discussed. Rupture of a splenic artery aneurysm (SAA) into the splenic vein and formation of a fistulous tract between them consist the underlying pathology. The authors emphasize the positive impact of transcatheter arterial embolization as an effective alternative to surgical intervention in the management of such vascular malformations.

CASE REPORT

A 50-year-old female was referred to our institution for management of an intermittent but intense epigastric pain and a feeling of gastric fullness that were associated with repeated episodes of haematemesis and melaena during the past twelve days. No history of abdominal (accidental or iatrogenic) trauma was reported. On the other hand, it was notable the fact that the patient had delivered successfully four labors between the ages of 26-40 years old.

Upon admission the patient's abdomen was distended and tender. The liver was not palpable and no signs of jaundice were observed. Abdominal auscultation revealed a systolic bruit located at the left flank. Ascites was also depicted. Full blood cell count exhibited markedly decreased values of hematocrit and hemoglobin. Coagulation studies and liver and renal function tests were not affected. Serological markers of hepatitis A, B, C, D were negative and no antibodies were detected. Urgent endoscopy showed multiple bleeding esophageal varices. The stomach was

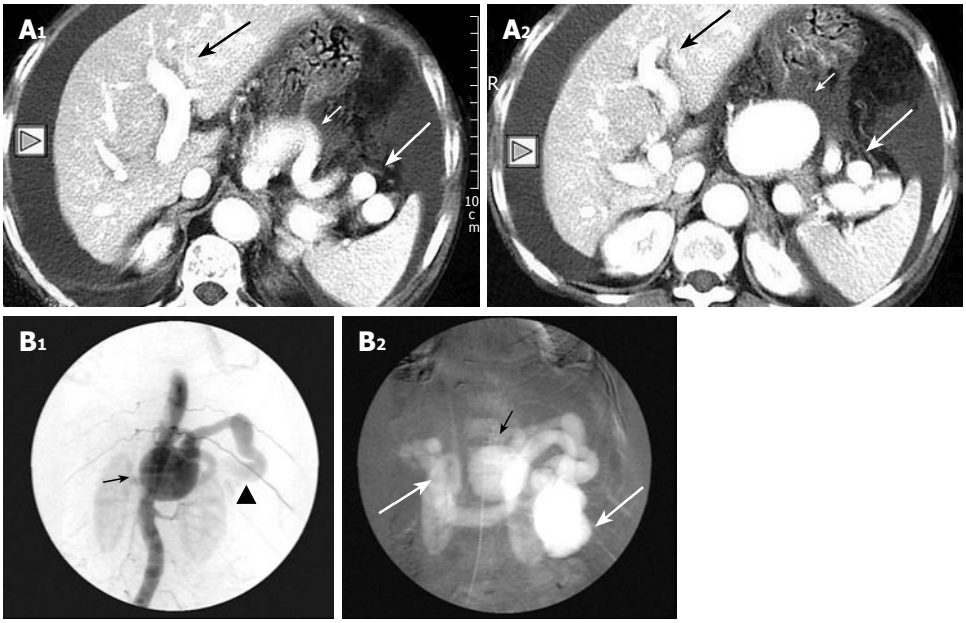

Figure 1 **A**: Post contrast CT scan reveals a tortuous and aneurysmatic splenic artery of maximum diameter of approximately 52 mm (short white arrow) associated with dilated vessels at the splenic hilum (long white arrow) and early opacification of the portal axis (long black arrow). In addition ascites is present as well (small triangle); **B**: Celiac angiogram confirms the presence of the splenic artery aneurysm (black arrow) in contiguity with the markedly dilated splenic vein and the premature and intense filling of the splenoportal trunk (black arrowhead and white arrows).

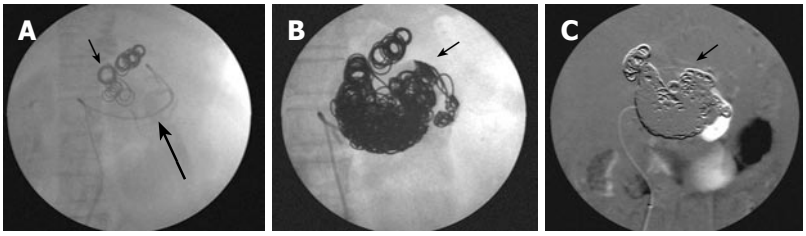

Figure 2 Selective transarterial catheterization (long black arrow) and embolization of the aneurysmal sac with numerous adequate metallic macrocoils (small black arrows) resulted in full occlusion of the sac and the fistulous tract enabling thus the reduction of the pressure in the splenoportal circulation.

found full of blood and the gastric mucosa was red irritated and edematous; findings consistent with mild portal gastritis. The endoscopic variceal ligation that immediately followed only controlled the bleeding temporarily.

Contrast enhanced CT scan of the upper abdomen demonstrated an aneurysmal dilated tortuous splenic artery of maximum diameter of 52 mm, an engorged splenic vein along with simultaneous opacification of the splenic vessels as well during the arterial phase (Figure 1A). Ascites was also confirmed. The aforementioned imaging features strongly supported the case of an SAA that was connected to the splenic vein through a fistula and caused an abrupt elevation of the portal pressure. Celiac and splenic arteriographies were carried out and showed a dilated splenic artery with a large aneurysm combined with premature portal and splenic vein filling and marked opacification of the splenoportal axis (Figure 1B).

The patient's condition remained unstable due to continuing bleeding and transcatheter embolization was scheduled. By using the transfemoral route the aneurysmal sac was accessed and subsequently detachment of adequate metallic macrocoils was performed resulting thus in full occlusion of the sac, cessation of the hyperkinetic portal flow and successful control of the gastro-intestinal bleeding (Figure 2).

The patient had an uncomplicated recovery and was discharged eight days later in stable clinical condition. At the time of this writing, 10 mo later the patient remains free of symptoms with no evidence of post-procedural recurrence or complications.

DISCUSSION

SAA was first described by Beaussier in 1770[5]. According to autopsy studies its prevalence ranges from 0.01% to 10.4%. SAAs are found incidentally on 0.78% of angiograms and in 7.1% of patients with cirrhotic portal hypertension[6]. Splenic artery accounts for the third most common abdominal vessel site that is affected by the aneurysmal disease after infra-renal aorta and iliac artery[7,8].

Splenic artery aneurysm formation relies upon structural incompetence of the connective tissue of the arterial wall to secure the integrity of the vessel lumen[5,6,8]. Associated risk factors include portal hypertension, connective tissue disorders, congenital abnormalities, trauma and infection[6]. Unlike other true visceral aneurysms atherosclerosis does not play a leading role in splenic artery aneurysmal dilatation. They are most commonly encountered among females (female to male ratio: 4/1) especially the multipara[6].

It has been postulated that increased visceral blood flow and gestational hormones during pregnancy alter pathologically the structure of the arterial wall by causing fragmentation of the elastic fibers of the media and loss of smooth muscle[6,8]. As parity increases these factors have a cumulative effect predisposing thus to aneurysmal formation[6]. Hence, the fact that this patient had four successful pregnancies can justify the progressive growth of a splenic artery aneurysm.

Splenic artery aneurysm may present as an incidental finding on imaging modalities or with abdominal pain[6].

It has been also reported a lifetime risk of rupture of 2%-10% with an associated mortality of 25%[9]. These rates increase remarkably among pregnant women whereas the estimated risk of rupture can reach up to 50% with related maternal and fetal mortality of 70% and 90% respectively[6]. The rupture may take place either into the gastrointestinal tract and peritoneal cavity or into the splenic vein resulting in splenic arteriovenous fistula development.

Splenic arteriovenous fistulas represent an unusual clinical entity that was first reported by Wiegert in the 19th century[1-4]. Their majority occurs more commonly after rupture of an SAA into the corresponding vein but their origin can be congenital, traumatic (iatrogenic or accidental) or even infectious[1-4].

Despite the fact that SAVFs may stay free of symptoms for a long period of time, the hemodynamic changes that develop owing to the arteriovenous shunt may lead to a sudden increase of portal vein pressure. In contrast, chronic liver disease follows a more time-consuming process before ending in portal hypertension[1].

The clinical profile of splenic arteriovenous fistula mainly consists of upper abdominal pain, gastro-intestinal bleeding and secretory diarrhea[1-4]. These features are attributed to the abrupt increase of the mesenteric venous pressure[1,3]. An abdominal bruit, usually of the "machinery" type, is often heard over the left flank[1,3,4] and although its presence can raise a high index of clinical suspicion, it is encountered only in 30% of cases[4].

Although abdominal ultrasound combined with the Color Doppler technology can document blood flow abnormalities, splenomegaly and exclude liver parenchymal disease, the established imaging modality in detecting SAVFs is selective celiac or splenic arteriography. The method localizes with accuracy the vascular abnormality and contributes greatly in the assessment of collateral flow pathways[10]. It is mostly recommended in cases with sudden onset of portal hypertension, abdominal bruit and absence of chronic liver disease[4]. The patient's medical history should be always taken into account.

On the basis of a well-established diagnosis immediate intervention is rendered mandatory in order to avoid unfavorable complications that will adversely affect prognosis. Splenic artery aneurysms should be promptly treated in high risk patients with symptomatic or expanding aneurysms, women of childbearing age and also patients undergoing liver transplantation[5,11].

Traditionally surgical and or laparoscopic resection with or without splenectomy is employed in the treatment of SAAs[5-7,9-12]. However, this patient carried a substantial surgical risk owing to the continuing variceal bleeding and the high possibility of aneurysmal rupture, during surgery, due to its size and the elevated pressure in the splenoportal axis. In addition, surgical approach of SAVFs presents often with technical difficulties and potential hazards of failure because of the distal site of the lesion and the formation of adhesions and numerous portal collaterals[4]. As a result, endovascular techniques emerged as an alternative option. Aneurysmal exclusion using a stent graft was not considered as a feasible solution mostly due to the presence of the fistula and the tortuosity of the splenic artery. In this case successful control of the variceal bleeding and cessation of the hyperkinetic blood flow in the splenoportal axis were achieved by selective catheterization of the sac and detachment of adequate metallic coils that promote permanent thrombosis. Unlike surgical techniques transcatheter arterial embolization is a less invasive, relatively low-risk, rapid procedure that can be easily applied regardless of the location of the vascular malformation[4,12]. It does not necessitate splenectomy avoiding thus immunologic deficits and a lifelong risk of sepsis[13]. The aforementioned advantages of transcatheter arterial embolization over surgery support strongly its efficacy in treating safely patients with splenic artery aneurysm complicated with arteriovenous fistula[4,12,13].

REFERENCES

1 **Strassburg CP**, Bleck JS, Rosenthal H, Meyer HJ, Gebel M, Manns MP. Diarrhea, massive ascites, and portal hypertension: rare case of a splenic arterio-venous fistula. *Z Gastroenterol* 1996; **34**: 243-249

2 **Galler G,** Yim A, Emond JC, Kirch E, Yousefzadeh D, Baker AL. Splenic arteriovenous fistula. A rare lesion causing bleeding esophageal varices, ascites, and diarrhea. *Dig Dis Sci* 1996; **41**: 816-819

3 **McClary RD**, Finelli DS, Croker B, Davis GL. Portal hypertension secondary to a spontaneous splenic arteriovenous fistula: case report and review of the literature. *Am J Gastroenterol* 1986; **81**: 572-575

4 **Hung CF,** Tseng JH, Lui KW, Wan YL, Tsai CC, Shem CH, Wu CS. Intractable oesophageal variceal bleeding caused by splenic arteriovenous fistula: treatment by transcatheter arterial embolization. *Postgrad Med J* 1999; **75**: 355-357

5 **Mattar SG**, Lumsden AB. The management of splenic artery aneurysms: experience with 23 cases. *Am J Surg* 1995; **169**: 580-584

6 **Selo-Ojeme DO**, Welch CC. Review: spontaneous rupture of splenic artery aneurysm in pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2003; **109**: 124-127

7 **Pulli R,** Innocenti AA, Barbanti E, Dorigo W, Turini F, Gatti M, Pratesi C. Early and long-term results of surgical treatment of splenic artery aneurysms. *Am J Surg* 2001; **182**: 520-523

8 **Cressey D**, Reid MF. Splenic artery aneurysm rupture in pregnancy. *Int J Obstet Anesth* 1996; **5**: 103-104

9 **Kasir D,** Macierewicz J, Castillo-Rama M, Singh S, Mackinlay J. Stent-graft repair of proximal splenic artery aneurysm. *Eur J Vasc Endovasc Surg* 2003; **6**: 62-63

10 **de Perrot M**, Bühler L, Deléaval J, Borisch B, Mentha G, Morel P. Management of true aneurysms of the splenic artery. *Am J Surg* 1998; **175**: 466-468

11 **Schmittling ZC,** McLafferty R. Transcatheter embolization of a splenic artery aneurysm. *J Vasc Surg* 2004; **40**: 1049

12 **Kakizawa H,** Toyota N, Naito A, Fukuda H, Ito K. Superselective transcatheter arterial embolization of a traumatic splenic arteriovenous fistula. *Radiat Med* 2004; **22**: 364-367

13 **Maloo MK,** Burrows PE, Shamberger RC. Traumatic splenic arteriovenous fistula: splenic conservation by embolization. *J Trauma* 1999; **47**: 173-175

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PO Box 2345, Beijing 100023, China
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CASE REPORT

Treatment of solitary gastric carcinoid tumor by endoscopic polypectomy in a patient with pernicious anemia

Gurhan Kadikoylu, Irfan Yavasoglu, Vahit Yukselen, Esra Ozkara, Zahit Bolaman

Gurhan Kadikoylu, Zahit Bolaman, Internist, Hematologist, Adnan Menderes University Medical Faculty, Division of Hematology, Aydin, Turkey
Irfan Yavasoglu, Internist, Fellow in hematology, Adnan Menderes University Medical Faculty, Division of Hematology, Aydin, Turkey
Vahit Yukselen, Internist, Gastroenterologist, Adnan Menderes University Medical Faculty, Division of Gastroenterology, Aydin, Turkey
Esra Ozkara, Pathologist, Adnan Menderes University Medical Faculty, Division of Pathology, Aydin, Turkey
Correspondence to: Gurhan Kadikoylu, Adnan Menderes University Medical Faculty, Division of Hematology, 09100 Aydin, Turkey. gurhan@medscape.com
Telephone: +90-256-2120020 Fax: +90-256-2146495
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Abstract

Type I gastric carcinoid tumors result from hypergastrinemia in 1%-7% of patients with pernicious anemia. We diagnosed pernicious anemia in a 48-year-old female patient with complaint of fatigue for three months. She had no gastrointestinal symptoms. Endoscopic examination ot the upper gastrointestinal tract revealed atrophic gastritis and a polypoid lesion in the corpus of 3-4 mm in size. Endoscopic polypectomy was performed. Histopathological examination of the specimen revealed positive chromogranin A and synaptophysin stainings compatible with the diagnosis of a carcinoid tumor. Serum gastrin level was increased, urinary 5-hydroxyindoleacetic acid was within the normal range. There was no other symptom, sign, or laboratory finding of a carcinoid syndrome in the patient. No metastasis was found with indium-111 octreotide scan, computed tomographies of abdomen and thorax. Type I gastric carcinoid tumors are only rarely solitary and patients with tumors < 1 cm in size may benefit from endoscopic polypectomy.

Key words: Solitary; Pernicious anemia; Gastric carcinoid tumor; Endoscopic polypectomy

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INTRODUCTION

Pernicious anemia is characterized by type A (autoimmune) atrophic gastritis, megaloblastic anemia, and neurological and psychiatric findings. It is the most common cause of cobalamin deficiency due to a loss of acid-secreting parietal cells in the gastric body and fundus[1]. It is known that patients with pernicious anemia have a higher risk to develop gastrointestinal malignancies such as gastric adenocarcinoma and carcinoid tumors, or oesophageal squamous cell carcinoma[1-4].

Gastric carcinoid tumors, which arise from hyperplasia of enterochromaffin-like (ECL) cells in the gastric mucosa, comprise 1%-3% of gastric neoplasms[5-7]. Type I carcinoid tumors are associated with ECL cell hyperplasia, hypergastrinemia, achlorhydria, and chronic atrophic gastritis, with or without pernicious anemia[8,9]. Type I carcinoid tumors are detected approximately in 1%-7% of the patients with pernicious anemia[1,3,9,10]. Most of the gastric carcinoid tumors are multifocal, solitary tumors are rare[3,11-14].

This report describes the treatment of a solitary gastric carcinoid tumor by endoscopic polypectomy in a patient with pernicious anemia.

CASE REPORT

A 48-year-old female patient who was suffering from fatigue for three months was admitted to the hospital. She had no gastrointestinal symptoms, and she negated alcohol abuse. Physical examination revealed pallor, jaundice, and atrophic glossitis. A grade-2 systolic ejection murmur was auscultated on the pulmonary area. Laboratory findings were a hemoglobin level of 59 g/L, a white blood cell count of 2400/mm^3, a platelet count of 76000/mm^3, a mean erythrocyte volume of 99 fL, and a reticulocyte count of 2.5%. Peripheral blood smear examination revealed 42% neutrophils, 40% lymphocytes, 12% monocytes, 6% eosinophils, and 3% normoblasts. Moreover, macrocytosis, aniso-poikilocytosis, Howell-Jolly bodies, Cabot ring in erythrocytes, and hypersegmentation in neutrophils were observed. Bone marrow aspiration revealed erythroid hyperplasia (E/M ratio: 6/1), evident megaloblastic changes, and nucleo-

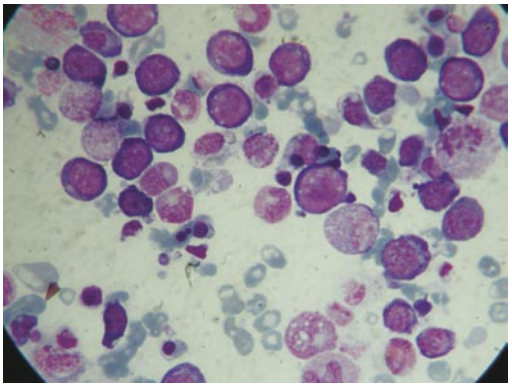

Figure 1 Megaloblastic changes in the bone marrow.

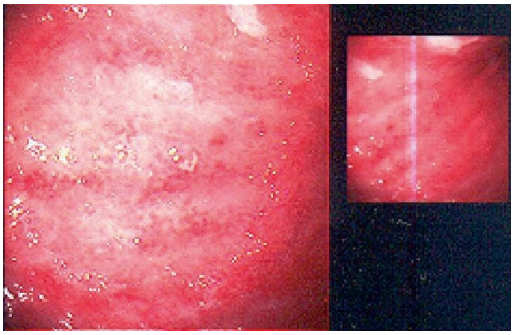

Figure 2 Polypoid appearance and atrophic gastritis at endoscopy.

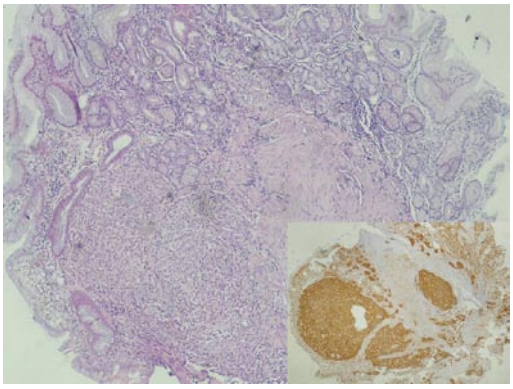

Figure 3 Carcinoid tumor positive stained with hematoxylin-eosin A and synaptophysin (x 40).

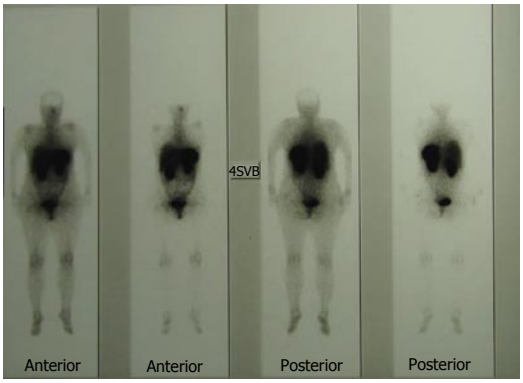

Figure 4 Indium-111 octreotide scan of the patient.

cytoplasmic dissociation in normoblasts (Figure 1). While iron parameters were normal, the levels of vitamin B_{12} and homocsyteine were 62 ng/L (N: 200-900 ng/L) and 91 μmol/L (N: 0-12 μmol/L), respectively. Anti-parietal cell antibody was positive. The biochemical values were normal except those for lactate dehydrogenase 1635 IU/L, total bilirubin 44 mg/L, and indirect bilirubin 34 mg/L. Thyroid function tests were normal, too. By performing upper gastrointestinal tract endoscopy, atrophic gastritis in fundus and corpus and a polypoid lesion in the corpus of 3-4 mm in size were seen (Figure 2). *H pylori* were not detected with rapid urease test. Endoscopic polypectomy was performed. By histopathological examination, a carcinoid tumor was diagnosed, mainly on the basis of positive stainings for chromogranin A and synaptophysin. *H pylori* was negative (Figure 3). The levels of fasting serum gastrin and 24 h urinary 5-hydroxyindoleacetic acid were 2000 ng/L and 12.6 μmol (N: 10.4-31.2 μmol), respectively. Spiral computed tomographies of thorax and abdomen were normal. Indium-111 octreotide scan revealed a normal distribution of tracer activity (Figure 4). Electrocardiography, transthoracic Doppler echocardiography, chest X-ray, and gastric endoscopic ultrasonography were normal. Endoscopic re-examination of the stomach was performed and multiple biopsies were obtained from the polypectomy site. The second pathological examination failed to detect any remnant of the carcinoid tumor or neuroendocrine cell hyperplasia. Treatment with vitamin B_{12} at a dose of 1000 μg/d orally was started[15]. After 6 mo follow-up, the patient was well, and her whole blood counts and the vitamin B_{12} level were normal.

DISCUSSION

Type I gastric carcinoid tumors result from hypergastrinemia in patients with pernicious anemia. Hypergastrinemia is the result of achlorhydria and atrophic gastritis. Hypergastrinemia acts as a trophic factor for ECL cells, resulting in hyperplasia in gastric mucosa[6,9-11,16,17]. Serum gastrin levels usually range from 740-4000 ng/L in those patients[17]. Although most of those patients are asymptomatic, dyspepsia, pain, nausea, unexplained weight loss, gastrointestinal bleeding, or anemia may be seen[11,13,17]. These carcinoid tumors are usually localized in the gastric fundus or corpus and are less than 2 cm in diameter. Often, they are multifocal. Nodal and hepatic metastasis occur in 2% of the patients[3,11,12,14,17]. Solitary gastric carcinoid tumors that like our patient were reported infreq uently[11,13,14,17]. Type II gastric carcinoid tumors, a very rare type, occurs in patients with Zollinger-Ellison syndrome. Most of carcinoid tumors are associated with multiple endocrine neoplasia (MEN) type I. Hypergastrinemia and ECL cell hyperplasia are seen in type II gastric carcinoid tumors. They are multifocal, and their sizes are between 1-2 cm. Local lymph node metastasis can produce[5,6,11]. Type III gastric carcinoid tumors are sporadic, they are not associated with hypergastrinemia, and, in contrast to types I and II, they are invasive large solitary tumors. Carcinoid tumors larger than 3 cm cause metastasis in 66%

of the patients while metastasis is detected in only 10% of patients with single tumors smaller than 1 cm[11,17,18].

In our patient, pernicious anemia was diagnosed because of macrocytic anemia, megaloblastic changes in the bone marrow, a decreased vitamin B_{12} level, an increased homocsyteine level, and atrophic gastritis. The pathological examination of the polyp revealed a solitary gastric carcinoid tumor. Serum gastrin level was high, and urinary 5-hydroxyindoleacetic acid level was normal. There were not symptoms, signs, or laboratory findings of a carcinoid syndrome in our patient. Because of hypergastrinemia in the absence of a Zollinger-Ellison syndrome or MEN type I, the final diagnosis was type I gastric carcinoid tumor.

The best approach modality to the patients with type I gastric carcinoid tumors is endoscopic resection especially in tumors smaller than 1 cm[17,19]. Antrectomy/total gastrectomy is recommended in cases with multiple tumors larger than 1 cm, and with abdominal symptoms such as unexplained weight loss, or aggravation of anemia[6,17,19,20].

We performed endoscopic polypectomy and re-evaluated the patient endoscopically with repeated biopsies. Metastasis was not detected in computed tomographies, pathological examinations, and octreotide scan. Thus, antrectomy/total gastrectomy was not performed. Annual follow-up with endoscopy and computed tomography are planned for the patient. In pernicious anemia, the incidence of gastric carcinoid tumors is increasing as a result of the careful endoscopic and pathological examinations. Type I solitary gastric carcinoid tumors are infrequently detected solitary and endoscopic polypectomy may cure the patient if the tumor is smaller than 1 cm. A follow-up program should be scheduled for patients who underwent polypectomy for any metastasis.

REFERENCES

1 **Carmel R**. Megaloblastic anemias: Disorders of impaired DNA synthesis. In: Greer JP, Foerster J, Lukens JN, Rodgers GM, Paraskevas F, Glader B, eds. Wintrobe's Clinical Hematology. 11th edition. Philadelphia: Lippincott Williams-Wilkins, 2004: 167-195

2 **Ye W**, Nyrén O. Risk of cancers of the oesophagus and stomach by histology or subsite in patients hospitalised for pernicious anaemia. *Gut* 2003; **52**: 938-941

3 **Sjöblom SM**, Sipponen P, Järvinen H. Gastroscopic follow up of pernicious anaemia patients. *Gut* 1993; **34**: 28-32

4 **Kokkola A**, Sjöblom SM, Haapiainen R, Sipponen P, Puolakkainen P, Järvinen H. The risk of gastric carcinoma and carcinoid tumours in patients with pernicious anaemia. A prospective follow-up study. *Scand J Gastroenterol* 1998; **33**: 88-92

5 **Rindi G**, Bordi C, Rappel S, La Rosa S, Stolte M, Solcia E. Gastric carcinoids and neuroendocrine carcinomas: pathogenesis, pathology, and behavior. *World J Surg* 1996; **20**: 168-172

6 **Kulke MH**. Neuroendocrine tumours: clinical presentation and management of localized disease. *Cancer Treat Rev* 2003; **29**: 363-370

7 **Attila T**, Santharam R, Blom D, Komorowski R, Koch TR. Multifocal gastric carcinoid tumor in a patient with pernicious anemia receiving lansoprazole. *Dig Dis Sci* 2005; **50**: 509-513

8 **Borch K**, Renvall H, Liedberg G. Gastric endocrine cell hyperplasia and carcinoid tumors in pernicious anemia. *Gastroenterology* 1985; **88**: 638-648

9 **Annibale B**, Azzoni C, Corleto VD, di Giulio E, Caruana P, D'Ambra G, Bordi C, Delle Fave G. Atrophic body gastritis patients with enterochromaffin-like cell dysplasia are at increased risk for the development of type I gastric carcinoid. *Eur J Gastroenterol Hepatol* 2001; **13**: 1449-1456

10 **Gilligan CJ**, Lawton GP, Tang LH, West AB, Modlin IM. Gastric carcinoid tumors: the biology and therapy of an enigmatic and controversial lesion. *Am J Gastroenterol* 1995; **90**: 338-352

11 **Binstock AJ**, Johnson CD, Stephens DH, Lloyd RV, Fletcher JG. Carcinoid tumors of the stomach: a clinical and radiographic study. *AJR Am J Roentgenol* 2001; **176**: 947-951

12 **Moneghini D**, Fontana MG, Villanacci V, Salerni B. [Multifocal gastric carcinoid associated with chronic type A atrophic gastritis. Analysis of 2 clinical cases]. *Ann Ital Chir* 2001; **72**: 89-92; discussion 92-93

13 **Sculco D**, Bilgrami S. Pernicious anemia and gastric carcinoid tumor: case report and review. *Am J Gastroenterol* 1997; **92**: 1378-1380

14 **Balthazar EJ**, Megibow A, Bryk D, Cohen T. Gastric carcinoid tumors: radiographic features in eight cases. *AJR Am J Roentgenol* 1982; **139**: 1123-1127

15 **Bolaman Z**, Kadikoylu G, Yukselen V, Yavasoglu I, Barutca S, Senturk T. Oral versus intramuscular cobalamin treatment in megaloblastic anemia: a single-center, prospective, randomized, open-label study. *Clin Ther* 2003; **25**: 3124-3134

16 **Qvigstad G**, Qvigstad T, Westre B, Sandvik AK, Brenna E, Waldum HL. Neuroendocrine differentiation in gastric adenocarcinomas associated with severe hypergastrinemia and/or pernicious anemia. *APMIS* 2002; **110**: 132-139

17 **Jordan PH Jr**, Barroso A, Sweeney J. Gastric carcinoids in patients with hypergastrinemia. *J Am Coll Surg* 2004; **199**: 552-555

18 **Modlin IM**, Tang LH. The gastric enterochromaffin-like cell: an enigmatic cellular link. *Gastroenterology* 1996; **111**: 783-810

19 **Spoelstra-de Man AM**, Wagenaar SS, van der Sluys Veer A, Brouwer CB. Relationship between pernicious anaemia and gastric neuroendocrine cell disorders. *Neth J Med* 2000; **56**: 56-62

20 **Modlin IM**, Lye KD, Kidd M. Carcinoid tumors of the stomach. *Surg Oncol* 2003; **12**: 153-172

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CASE REPORT

Computed tomographic findings of trichuriasis

Naime Tokmak, Zafer Koc, Serife Ulusan, Ismail Soner Koltas, Nebil Bal

Naime Tokmak, Zafer Koc, Serife Ulusan, Başkent University, Adana Teaching and Medical Research Center, Department of Radiology, Adana, Turkey
Ismail Soner Koltas, Çukurova University, Faculty of Medicine, Department of Parasitology, Adana, Turkey
Nebil Bal, Başkent University, Adana Teaching and Medical Research Center, Department of Pathology, Adana, Turkey
Correspondence to: Dr. Zafer Koc, Başkent Universitesi Adana Hastanesi Dadaloğlu Mah. Serin Evler 39. Sok. No: 6 01250 Yüreğir, Adana, Turkey. koczafer@gmail.com
Telephone: +90-322-3272727-1025 Fax: +90-322-3271270
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Abstract

In this report, we present computed tomographic findings of colonic trichuriasis. The patient was a 75-year-old man who complained of abdominal pain, and weight loss. Diagnosis was achieved by colonoscopic biopsy. Abdominal computed tomography showed irregular and nodular thickening of the wall of the cecum and ascending colon. Although these findings are nonspecific, they may be one of the findings of trichuriasis. These findings, confirmed by pathologic analysis of the biopsied tissue and Kato-Katz parasitological stool flotation technique, revealed adult *Trichuris*. To our knowledge, this is the first report of colonic trichuriasis indicated by computed tomography.

Key words: *Trichuris trichiura*; Colitis; Large bowel; Imaging findings; Computed tomography

Tokmak N, Koc Z, Ulusan S, Koltas IS, Bal N. Computed tomographic findings of trichuriasis. *World J Gastroenterol* 2006; 12(26): 4270-4272

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INTRODUCTION

Trichuris trichiura (T. trichiura), a whipworm, is the third most common nematode worldwide after *Ascaris* and *Enterobius*[1]. First described by Roederer in 1761, the whipworm is found primarily in tropical climates characterized by poor sanitation[1]. Whipworm infection usually causes no clinical symptoms, although a severe infection can cause abdominal pain, diarrhea, constipation, weight loss, and anemia[2-5].

In the patient described in this report, we identified irregular and nodular marked thickening of the wall of the cecum and ascending colon as a CT finding of trichuriasis. To our knowledge, this is the first report of colonic trichuriasis revealed by that modality. A follow-up CT scan showed that the colonic manifestations of trichuriasis had resolved six months after the conclusion of the medical treatment.

CASE REPORT

A 75-year-old man with suspected colon carcinoma was referred to our radiology unit for evaluation by computed tomography (CT). He complained of abdominal pain, constipation, and a weight loss of 5 kg in the prior two months. Physical examination revealed mild tenderness in the right lower abdomen. The patient's white blood cell count was 13.9 × 10^9/L (reference intervals, 5-11 × 10^9/L) without eosinophilia. The results of other laboratory tests (including biochemical analysis) were within the normal range with the exception of moderate anemia (hemoglobin level, 90 g/L; reference intervals, 140-180 g/L).

CT scans were obtained using a four-channel MDCT scanner (Sensation 4; Siemens, Erlangen, Germany). A mixture of 1000 mL water and 40 mL contrast material was used for bowel opacification. Portal phase CT scans (2.5 mm collimation, 12.5 mm/0.5 s table speed) were acquired at 60 s after contrast material injection.

CT scan of the abdomen showed irregular and nodular marked thickening of the wall of the cecum and ascending colon (Figure 1A, B). Endoscopic examination revealed multiple ulcerated mucosal lesions in the ascending colon. The cecum could not be properly examined because of intestinal content. The results of pathologic analysis of the biopsied tissue, which was obtained colonoscopically from the ascending colon, revealed adult *Trichuris* and the signs of active colitis (Figure 2); these findings were confirmed by Kato-Katz parasitological stool flotation technique[6]. The patient was subsequently treated with mebendazole 100 mg twice daily for 3 d. On follow-up at the end of the treatment clinical findings had resolved, and a repeat CT scan obtained 6 mo after the treatment revealed a colonic wall thickness within the normal range (Figure 3) and no *T. trichiura* eggs were seen in any stool specimens.

DISCUSSION

Trichuriasis is an intestinal infection found in human beings, which is caused by *T. trichiura*, more commonly known as whipworm because of its whip-like appearance.

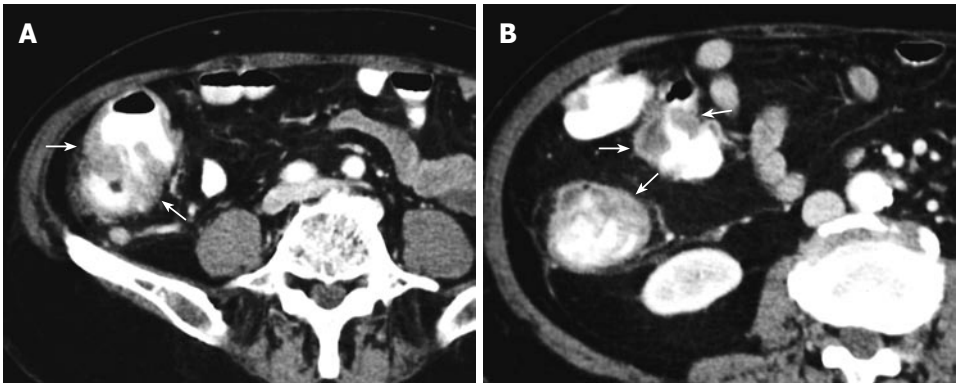

Figure 1 Computed tomographic scans (**A**, **B**) of the abdomen. Note the irregular and nodular thickened wall of the cecum and the ascending colon (arrows).

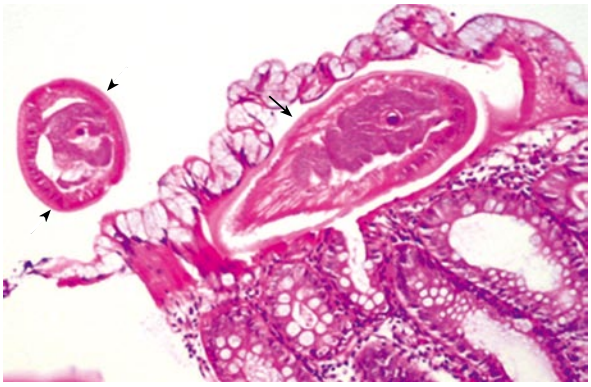

Figure 2 Pathologic examination reveals transverse section of anterior portion of *Trichuris trichiura* (arrow) embedded in superficial colonic mucosa and free body part of *Trichuris trichiura* (arrowheads) extending into the lumen of the colon.

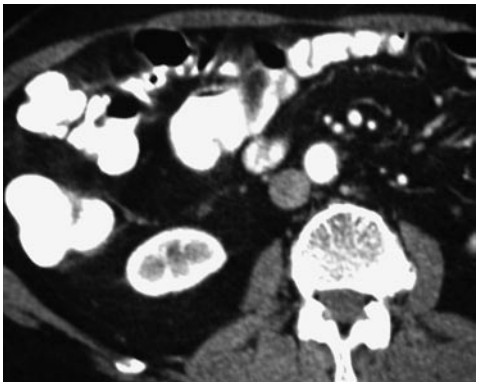

Figure 3 Computed tomographic scan obtained 6 mo after the treatment show a normal colonic wall thickness.

It is characterized by the invasion of the colonic mucosa by the adult *Trichuris* and produces minor inflammatory changes at the sites of localization. Trichuriasis remains a health problem in certain geographic areas. Infection is acquired by the ingestion of embryonated eggs from contaminated drinking water and food. The eggs hatch in the small intestine, and the larvae then enter the intestinal crypts[1]. After a brief period of maturation, the larvae may migrate to the proximal colon, where they reside and over 1 to 3 mo mature into adults[1,2,6,7]. The adult *T. trichiura* is a threadlike worm 30 to 50 mm in length. *T. trichiura* penetrates the intestinal mucosa and frequently triggers minor inflammatory changes in the cecum, appendix, and terminal ileum[7]. Diagnosis is usually based on the identification of typical *T. trichiura* eggs in stool specimens[6]. Adult whipworm is rarely seen during colonoscopy and colonoscopy can directly diagnose trichuriasis, confirming the threadlike form of worms with an attenuated end[7-11]. Literature research revealed that imaging findings sometimes similar with a tumor of the colon because of tumor-like appearance of the granulomatous tissue reaction due to *T. trichiura*[12].

A few reports have described the radiographic appearance of *T. trichiura* on double-contrast barium enema[13]. Characteristic findings revealed by that modality have included multiple tiny target-shaped or pinwheel-shaped collections of barium that are associated with peculiar s-shaped filling defects thought to be consistent with the shape of the adult male *T. trichiura*[13]. Colonic wall thickening is a common and nonspecific finding that occurs in many other conditions such as infections, inflammatory bowel disease and carcinoma. However, knowledge of this entity is helpful for differential diagnosis in the endemic areas of trichuriasis.

We concluded that colonoscopy with biopsy is the gold standard for such disease like trichuriasis. Irregular and nodular thickening of the cecum and the ascending colon on CT scan may be one of the findings of trichuriasis. Although that finding is nonspecific, parasitic infections including trichuriasis should be considered as differential diagnoses, especially in areas in which such infection is endemic.

REFERENCES

1 **Monroe LS**. Gastrointestinal parasites. In : Haubrich WS, Schaffner F, Berk JE, eds. Bockus Gastroenterology. 5th ed. Philadelphia: Saunders, 1995: 3113-3196
2 **Fishman JA**, Perrone TL. Colonic obstruction and perforation due to Trichuris trichiura. *Am J Med* 1984; **77**: 154-156
3 **Bahon J**, Poirriez J, Creusy C, Edriss AN, Laget JP, Dei Cas E. Colonic obstruction and perforation related to heavy Trichuris trichiura infestation. *J Clin Pathol* 1997; **50**: 615-616
4 **Huang NC**, Fang HC, Chou KJ, Chung HM. Trichuris trichiura: an unusual cause of chronic diarrhoea in a renal transplant patient. *Nephrol Dial Transplant* 2003; **18**: 2434-2435
5 **Hong ST**, Lim HS, Kim DH, Kim SJ. A case of gastroenteritis associated with gastric trichuriasis. *J Korean Med Sci* 2003; **18**: 429-432
6 **Santos FL**, Cerqueira EJ, Soares NM. Comparison of the thick

smear and Kato-Katz techniques for diagnosis of intestinal helminth infections. *Rev Soc Bras Med Trop* 2005; **38**: 196-198
7 **Chandra B**, Long JD. Diagnosis of Trichuris trichiura (whipworm) by colonoscopic extraction. *J Clin Gastroenterol* 1998; **27**: 152-153
8 **Davis M**, Matteson A, Williams WC. Radiographic and endoscopic findings in human whipworm infection (Trichuris trichiura). *J Clin Gastroenterol* 1986; **8**: 700-701
9 **Joo JH**, Ryu KH, Lee YH, Park CW, Cho JY, Kim YS, Lee JS, Lee MS, Hwang SG, Shim CS. Colonoscopic diagnosis of whipworm infection. *Hepatogastroenterology* 1998; **45**: 2105-2109
10 **Yoshida M**, Kutsumi H, Ogawa M, Soga T, Nishimura K, Tomita S, Kawabata K, Kinoshita Y, Chiba T, Fujimoto S. A case of Trichuris trichiura infection diagnosed by colonoscopy. *Am J Gastroenterol* 1996; **91**: 161-162
11 **Lorenzetti R**, Campo SM, Stella F, Hassan C, Zullo A, Morini S. An unusual endoscopic finding: Trichuris trichiura. Case report and review of the literature. *Dig Liver Dis* 2003; **35**: 811-813
12 **Kojima Y**, Sakuma H, Izumi R, Nakagawara G, Miyazaki I, Yoshimura H. A case of granuloma of the ascending colon due to penetration of Trichuris trichiura. *Gastroenterol Jpn* 1981; **16**: 193-196
13 **Manzano C**, Thomas MA, Valenzuela C. Trichuriasis. Roentgenographic features and differential diagnosis with lymphoid hyperplasia. *Pediatr Radiol* 1979; **8**: 76-78

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CASE REPORT

Delayed hemorrhage from hepatic artery after ultrasound-guided percutaneous liver biopsy: A case report

Fen-Yu Ren, Xi-Xu Piao, Ai-lian Jin

Fen-Yu Ren, Xi-Xu Piao, Ai-lian Jin, Department of Gastroenterology and Hepatology, Yanbian University Hospital, Yanji 133000, Jilin Province, China
Correspondence to: Fen-Yu Ren, MD, PhD, Department of Gastroenterology and Hepatology, Yanbian University Hospital, Yanji 133000, Jilin Province, China. fenyu65@yahoo.com.cn
Telephone: +86-433-2660061 Fax:+86-433-2513610
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Abstract

Percutaneous liver biopsy is considered one of the most important diagnostic tools to evaluate diffuse liver diseases. Pseudoaneurysm of hepatic artery is an unusual complication after ultrasound-guided percutaneous liver biopsy. Delayed hemorrhage occurs much less frequently. We report a case of pseudoaneurysm of the hepatic artery of a 46-year-old man who was admitted for abdominal pain after 4 d of liver biopsy. The bleeding was controlled initially by angiographic embolization. However, recurrent bleeding could not be controlled by repeat angiography, and the patient died 4 d after admission from multiorgan failure. The admittedly rare possibility of delayed hemorrhage should be considered whenever a liver biopsy is performed.

Key words: Hemorrhage; Hepatic artery; Percutaneous liver biopsy

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INTRODUCTION

Percutaneous liver biopsy is considered one of the most important diagnostic tools to evaluate diffuse liver diseases. The most common complication of percutaneous liver biopsy is bleeding. The major complication and mortality rates are about 2%-4% and 0.01%-0.33% respectively[1]. Ultrasonography-guided percutaneous liver biopsy has been shown to increase the diagnostic yield and significantly decrease complications even on outpatients[2-4]. The less common complication of delayed bleeding after percutaneous liver biopsy, has a higher mortality associated with late recognition[5]. Pseudoaneurysms of the hepatic artery initially should be managed with angiographic embolization, reserving surgical intervention for failure of embolization[6]. Here we report a case of fatal delayed hemorrhage from pseudoaneurysm of the hepatic artery after percutaneous liver biopsy. The diagnosis was confirmed by ultrasound and angiography.

CASE REPORT

A 46-year-old man with genotype 1b chronic hepatitis C virus (HCV) infection was evaluated for progressive fatigue and elevated aminotransferases. The hemoglobin, prothrombin time, bleeding time, and platelet count were normal. Abdominal ultrasound was normal and ultrasonography showed no focal hepatic lesion or evidence of cirrhosis. He received a liver biopsy to evaluate the pathologic change before interferon therapy. A percutaneous liver biopsy was conducted under ultrasonographic guidance with a 2.8-mm Menghini-type aspiration needle with one pass. Chronic hepatitis was diagnosed based on histopathological assessment according to a scoring system that includes semi-quantitative assessment of liver disease grading and staging[7]. Laboratory data and liver biopsy revealed moderate activity (grade 2/4, stage 2/4). After liver biopsy, transient hypotension was noted during the first two hours of in-hospital observation and the patient was discharged 6 h later.

Four days later, the patient complained of right upper quadrant pain radiating to the right shoulder. The abdomen was soft with normal bowel sounds and mild right upper quadrant tenderness without rebound tenderness. The patient denied abdominal trauma. Laboratory results including white blood cell count (WBC): 5.5 × 10^9/L; platelet count (PCT): 204 × 10^9/L; hematocrit (HCT): 47%; prothrombin time international normalized ratio (INR): 1.0; aspartate aminotransferase (AST): 107 IU/L; alanine aminotransferase (ALT): 145 IU/L; total bilirubin (TBIL): 16 μmol/L: alkaline phosphatase (ALP): 128IU/L; BUN and serum creatinine (CRE): were normal. His blood pressure (BP) was 19.3/11.3 kPa, and heart rate (HR) 69 beats/min. The diagnosis of pseudoaneurysm was established 4 d after the percutaneous liver biopsy by abdominal ultrasonography. The patient suddenly became hemodynamically unstable

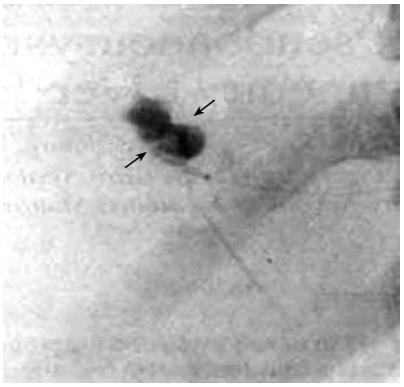

Figure 1 Angiography of the right hepatic artery. Pseudoaneurysm (arrows) in the right hepatic artery branch.

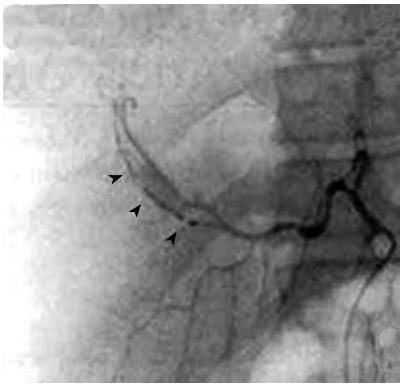

Figure 2 Platinum coils (arrows) are seen filling the feeding branch. Angiography obtained after embolization of the branch leading to and from the pseudoaneurysm.

with an HR 135 beats/min and BP 10.7/5.3 kPa 13 h after admission, which was over 5 d after the percutaneous liver biopsy. The repeat HCT was 39%. The patient was resuscitated and underwent immediate angiography, which showed a pseudoaneurysm in the right hepatic artery branch (Figure 1). The pseudoaneurysm measured approximately 1.8 cm × 2.1 cm. The patient was treated with embolization performed by multiple straight of the right hepatic artery branch (Figure 2). The angiography was repeated 28 h after admission and revealed continued extravasation of blood from the right lateral subcapsular location. These demonstrated new areas of active arterial extravasation with possible complex laceration of the right hepatic lobe. The laboratory tests obtained 33 h after admission showed INR 2.1; ALT: 3970 IU/L; AST: 4910 IU/L; ALP: 290 IU/L; TBIL: 32 μmol/L; albumin (ALB): 17 g/L, CRE: 230 μmol/L; and BUN: 19 mol/L. Soon afterward, the patient received surgery, which revealed right hepatic lobe laceration with hemorrhage. After surgery, the patient's condition continued to deteriorate with a decreasing HCT. Repeated ultrasound of the liver revealed the interval development of multiple cystic lesions in the right hepatic lobe compatible with intrahepatic hematomas. No further surgical intervention was performed because of the diffuse nature of the bleeding. The patient died 4 d after admission from multiorgan failure.

DISCUSSION

Outpatient percutaneous liver biopsy is a common practice in the differential diagnosis and treatment of chronic liver disease. The major complication and mortality rates were about 2%-4% and 0.01%-0.33% respectivery[1]. According to the recent National Institutes of Health (NIH) Consensus Conference on the management of chronic hepatitis C, liver biopsy is recommended before therapy to assess the severity and activity of the liver disease[8]. Treatment with interferon is recommended if the liver biopsy shows evidence of septal fibrosis and/or moderate-to-severe necroinflammatory changes. More recent view on the issue of performing a liver biopsy in hepatitis C patients advises to perform a liver biopsy in genotype 1 patients, but usefulness of histologic assessment in genotypes 2 or 3 is debated, since these patients have a very good virological response[9]. At the best, liver biopsy is supported by a grade III recommendation[9]. Early bleeding within hours can result from intrahepatic hematoma or laceration of the liver capsule[1]. Previous studies showed an overall complication rate of 0.28% and fatality rate of 0.03% among 189 085 liver biopsies[10]. Other reports showed, there were 3 deaths (0.004%) in 68 276 and 19 deaths (0.052%) in 36 786 liver biopsies[11]. Factors associated with an increased risk of hemorrhage included increasing age, number of biopsy passes and presence of malignancy in the liver[1,12]. The risk of hemorrhage or complication[13] was not related to the biopsy needle type or diameter of the needle[14].

Pseudoaneurysms more commonly occur in patients who are post liver transplant or post cholecystectomy[15,16]; only 11 pseudoaneurysms occur among 1211 patients undergoing multiple liver biopsies after liver transplantation, whereas only 2 (0.17%) cases were attributed to percutaneous liver biopsy[17]. This case report demonstrated an unusual complication of hepatic artery pseudoaneurysm from percutaneous liver biopsy. The use of teep mattress sutures for initial control of bleeding is preferred[18]. After percutaneous liver biopsy with liver diseases, most patients experiencing these events were asymptomatic, and angiographic defects were found in 61% of angiography performed within 1 wk but in 11% of angiography done after more than 1 wk[16]. The present case showed that clinical features of pseudoaneurysms may range from asymptomatic to hemobilia to massive delayed fatal hemorrhage. Ultrasound with Doppler can be used to diagnose pseudoaneurysm and to evaluate portal blood flow. A celiac or selective hepatic angiography will demonstrate the actual location and size of the pseudoaneurysm[16,19]. A few previous studies showed that embolization of a hepatic artery pseudoaneurysm had a success rate of 97%, and the occlusion of the more proximal hepatic artery caused by dissection or thrombosis may necessitate a direct percutaneous approach[15]. Both transarterial and direct percutaneous accesses to pseudoaneurysms have also been successfully employed[15]. However, direct embolization of the pseudoaneurysmm, allowing patency of the hepatic artery, has also been reported[15]. In this case, bleeding from pseudoaneurysm of the hepatitc artery was controlled initially with embolization, but laboratory tests performed after embolization suggest that the patient apparently had ischemic hepatitis and continued to deteriorate from rebleeding. The patient died 4 d after admission from

multiorgan failure. Ischemic hepatitis or shock liver is defined as an extensive hepatocellular necrosis associated with a decrease in hepatic perfusion due to systemic hypotension. Serum aminotransferase levels (ALT and AST) increased rapidly after the ischemic episode and peaked within 1 to 3 d to at least 20 times the upper normal limit. After recovery, aminotransferases returned to near normal levels in 7-10 d of the initial insult[20].

In conclusion, delayed fatal hemorrhage from pseudoaneurysm of hepatic artery is an unusual complication after percutaneous liver biopsy. Pseudoaneurysms should initially be managed with angiographic embolization[6], and surgical intervention may be needed if embolization fails.

REFERENCES

1 **McGill DB**, Rakela J, Zinsmeister AR, Ott BJ. A 21-year experience with major hemorrhage after percutaneous liver biopsy. *Gastroenterology* 1990; **99**: 1396-1400

2 **Younossi ZM**, Teran JC, Ganiats TG, Carey WD. Ultrasound-guided liver biopsy for parenchymal liver disease: an economic analysis. *Dig Dis Sci* 1998; **43**: 46-50

3 **Farrell RJ**, Smiddy PF, Pilkington RM, Tobin AA, Mooney EE, Temperley IJ, McDonald GS, Bowmer HA, Wilson GF, Kelleher D. Guided versus blind liver biopsy for chronic hepatitis C: clinical benefits and costs. *J Hepatol* 1999; **30**: 580-587

4 **Rossi P**, Sileri P, Gentileschi P, Sica GS, Forlini A, Stolfi VM, De Majo A, Coscarella G, Canale S, Gaspari AL. Percutaneous liver biopsy using an ultrasound-guided subcostal route. *Dig Dis Sci* 2001; **46**: 128-132

5 **Reichert CM**, Weisenthal LM, Klein HG. Delayed hemorrhage after percutaneous liver biopsy. *J Clin Gastroenterol* 1983; **5**: 263-266

6 **Croce MA**, Fabian TC, Spiers JP, Kudsk KA. Traumatic hepatic artery pseudoaneurysm with hemobilia. *Am J Surg* 1994; **168**: 235-238

7 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520

8 Management of hepatitis C. *NIH Consens Statement* 1997; **15**: 1-41

9 **Strader DB**, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147-1171

10 **Garcia-Tsao G**, Boyer JL. Outpatient liver biopsy: how safe is it? *Ann Intern Med* 1993; **118**: 150-153

11 **Caturelli E**, Giacobbe A, Facciorusso D, Bisceglia M, Villani MR, Siena DA, Fusilli S, Squillante MM, Andriulli A. Percutaneous biopsy in diffuse liver disease: increasing diagnostic yield and decreasing complication rate by routine ultrasound assessment of puncture site. *Am J Gastroenterol* 1996; **91**: 1318-1321

12 **McVay PA**, Toy PT. Lack of increased bleeding after liver biopsy in patients with mild hemostatic abnormalities. *Am J Clin Pathol* 1990; **94**: 747-753

13 **Gilmore IT**, Burroughs A, Murray-Lyon IM, Williams R, Jenkins D, Hopkins A. Indications, methods, and outcomes of percutaneous liver biopsy in England and Wales: an audit by the British Society of Gastroenterology and the Royal College of Physicians of London. *Gut* 1995; **36**: 437-441

14 **Froehlich F**, Lamy O, Fried M, Gonvers JJ. Practice and complications of liver biopsy. Results of a nationwide survey in Switzerland. *Dig Dis Sci* 1993; **38**: 1480-1484

15 **Rivitz SM**, Waltman AC, Kelsey PB. Embolization of an hepatic artery pseudoaneurysm following laparoscopic cholecystectomy. *Cardiovasc Intervent Radiol* 1996; **19**: 43-46

16 **Tsai CH**, Mo LR, Chiou CY, Ko QY, Hwang HS, Hwang MH, Yang CT, Liang TK. Therapeutic embolization of post-cholecystectomy hepatic artery aneurysm. *Hepatogastroenterology* 1992; **39**: 158-160

17 **Tobben PJ**, Zajko AB, Sumkin JH, Bowen A, Fuhrman CR, Skolnick ML, Bron KM, Esquivel CO, Starzl TE. Pseudoaneurysms complicating organ transplantation: roles of CT, duplex sonography, and angiography. *Radiology* 1988; **169**: 65-70

18 **Hellekant C**. Vascular complications following needle puncture of the liver. Clinical angiography. *Acta Radiol Diagn* (Stockh) 1976; **17**: 209-222

19 **Landy P**, Edwards RD, Blomley M. Embolization of hepatic artery pseudo-aneurysm facilitated by selective injection of thrombin and thrombin-soaked coils. *Australas Radiol* 1995; **39**: 257-259

20 **Squella F**, Zapata R. [Ischemic hepatitis. Case report]. *Rev Med Chil* 2003; **131**: 659-664

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Raul J Andrade, Associate Professor
Department of Medicine, University of Malaga, Boulevar louis pasteur 32, málaga 29010, Spain

Josep M Bordas, MD
Department of Gastroenterology IMD, Hospital Clinic", Llusanes 11-13 at, Barcelona 08022, Spain

Jamie S Barkin, MD, Professor of Medicine, Chief
Sinai Medical Center Division of Gastroenterology, Mt. Sinai Medical Center, University of Miami, School of Medicine, 4300 Alton Road, Miami Beach, FL 33140, United States

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Department of Medicine II, University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

Lee Bouwman, Dr.
Leiden University Medical Centre, department of surgery, Albinusdreef 2 PO Box 9600, 230 RC Leiden, The Netherlands

Michael F Byrne , MD, Clinical Associate Professor
Division of Gastroenterology Vancouver General Hospital, 100-2647 Willow Street Vancouver BC V5Z 3P1, Canada

Byung Ihn Choi, Professor
Department of Radiology, Seoul National University Hospital, 28, Yeongeon-dong, Jongno-gu, Seoul 110-744, South Korea

Xian-Ming Chen, MD
Center for Basic Research in Digestive Diseases, Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, 200 First Street, SW, Rochester, MN 55905, United States

Inge Irma Depoortere, PhD
Centre for Gastroenterological Research, Gasthuisberg OandN, bus 701, Leuven 3000, Belgium

John Frank Di Mari, Assistant Professor
Internal Medicine, Gastroenterology ,9.138 MRB 301 University Blvd. Galveston, Texas 77555-1064, United States

Bijan Eghtesad, Dr., Associate Professor
Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland OH 44195, United States

Jiro Fujimoto, Professor
First Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

Mitsuhiro Fujishiro, Dr.
Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Valeria Ghisetti, Dr.
Laboratory of Microbiology, Molinette Hospital, Corso Bramante 88 / 90, 10126 Torino, Italy

Ming-Liang He, Associate Professsor
Faculty of Medicine , The Center for Emerging Infectious Diseases, The Chinese University of Hong Kong, Hong Kong, Chin

Shinn-Jang Hwang, Professor
Department of Family Medicine, Taipei Veterans General Hospital, VGH, 201, Shih-Pai Road, Section 2, 11217, Taiwan, China

Tetsuo Hayakawa, Emeritus Professor
Director general, Meijo Hospital, Meijo Hospital, Sannomaru 1-3-1, Naka-ku, Nagoya 460-0001, Japan

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Jamal A Ibdah, MD, PhD, Professor, Director
Division of Gastroenterology and Hepatology, University of Missouri at Columbia, One Hospital Drive, Columbia, MO 65212, United States

Toru Ishikawa, MD
Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Aydin Karabacakoglu, Dr., Assistant Professor
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Jae J Kim, MD, PhD, Associate Professor
Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Myung-Hwan Kim, Professor
Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-dong, Songpa-gu, Seoul 138-736, South Korea

Serdar Karakose, Dr., Professor
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Takashi Kanematsu, Professor
Division of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Zahariy Krastev, Professor
Department of Gastroneterology, Universiti Hospital "St. Ivan Rilski", #15, blvd "Acad. Ivan Geshov", Sofia 1431, Bulgaria

Kurt Lenz, Professor
Department of Internal Medicine, Konventhospital Barmherzige Brueder, A-4020 Linz, Austria

Peter Laszlo Lakatos, MD, PhD, Assistant Professor
1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Rene Lambert, Professor
International Agency for Research on Cancer, 150 Cours Albert Thomas, Lyon 69372 cedex 8, France

John M Mariadason, PhD, Assistant Professor
Department of Oncology, Albert Einstein College of Medicine, Montefiore Medical Center, Hofheimer Bldg. 413, 111 East 210th Street, Bronx, NY 10467, United States

Masatoshi Makuuchi, Professor
Department of Surgery, Graduate School of Medicine University of Tokyo, T Hepato-Biliary-Pancreatic Surgery Division Tokyo 113-8655, Japan

Johann Ockenga, Dr.
Gastroenterology, Hepatology and Endocrinology, Charite-Universitatsmedizin Berlin, Schumannstrasse 20/21, 10117 Berlin, Germany

Markus Peck-Radosavljevic, Professor
Department of Internal Medicine IV, Division of Gastroenterology and Hepatology, University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Eamonn M Quigley, Professor
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Jean Rosenbaum, Dr.
Inserm E362, Universite Victor Segalen Bordeaux 2, Bordeaux 33076, France

Steffen Rickes, Dr.
Department of Gastroenterology and Hepatology, University Hospital Magdeburg, Germany

Chifumi Sato, Professor
Department of Analytical Health Science, Tokyo Medical and Dental University, Graduate School of Health Sciences, 1-5-45 Yushima, Bunkyoku, Tokyo 113-8519, Japan

Michael Steer, Professor
Department of Surgery, Tufts-Nemc, 860 Washington St, Boston, Ma 02111, United States

Tadatoshi Takayama, Professor
Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan

Fritz von Weizsäcker, Professor
Department of Medicine Schlosspark-Klinik, Humboldt University, Heubnerweg 2, Berlin D-14059, Germany

Ian David Wallace, MD
Shakespeare Specialist Group, 181 Shakesperare Rd, Milford, Auckland 1309, New Zealand

Wai-Man Wong, MD
Department of Medicine, University of Hong Kong, St Paul's Hospital, 2 Eastern Hospital Road, Causeway Bay, Hong Kong, China

Jesus K Yamamoto-Furusho, Dr.
Gastroenterology, Instituto Nacional de Ciencias Medicas y Nutricion, Vasco de Quiroga 15, Col. seccion XVI, Mexico 14000, Mexico

Yoshio Yamaoka, MD, PhD, Associate Professor
Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, Texas 77030, United States

Xin-Xin Zhang, Professor
Department of Infectious Disease, Rui Jin Hospital, 197, Rui Jin Er Road, Shanghai 200025, China

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
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Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
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European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
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World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
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AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
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13-16 December 2006
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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

In press

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

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

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

No author given

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

Volume with supplement

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

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]

No volume or issue

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

Books

Personal author(s)

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

Chapter in a book (list all authors)

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

Author(s) and editor(s)

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

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour 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)

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

Patent (list all authors)

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

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